STUDIES ON THE VISUAL SYSTEM OF THE RAT

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SUMMARY

Minimal structural changes, not detected by qualitative assessment require quantitative methods for recognition. This work is concerned with the development of such a quantitative technique, and its application to minimal change situations, namely the visual cortices of rats with retinal dystrophy (Campbell strain) and of rats which had received a low grade retinotoxic insult.

As well as the anatomy and normal development, the literature survey included those factors which can influence the normal development and function of the visual system, and hence the quantitative data. The quantitation review demonstrated the diversity of approach of the various authors working in this field, and revealed how little quantitative data exists on the rat cerebrum. No reports could be found on visual system quantitation of either of the situations investigated in this work.

In the section on the problems of quantitation, the high technical demands and inherent errors are discussed, and the methods and correction factors to overcome these problems are detailed. Strict comparability of sections was attained by devising a method which ensured similar anatomical location in every case, despite great variation in brain size.

A technique was devised to measure the variable shrinkage of processing, a problem ignored by many authors. By means of this Reduction Factor, the exact volume of tissue in a section is known and quantitative data can be calculated in a standard comparable form.

From a total of 1,500 sections, after screening by ophthalmoscopy, post mortem examination, section thickness control and histological scrutiny, some 950 sections were selected for quantitation. The 950 sections, from 190 individuals provided the large sample, necessary because of individual variation.

The quantitative results are presented in graphical and statistical forms, subdivided by age, sex and strain. These results show the method revealed differences between the dystrophic (Campbell) and control (P.V.G.) visual cortices. The Campbell cortex has a smaller mean cell size, which is produced by two factors, namely an increase in small cell density and a decrease in the density of the largest cells. Ultrastructural and special light microscopic investigation identified these cells as microglia and neurones respectively.

These results, which have never previously been reported in the rat compare well with those produced in other species with visual deprivation and are consistent with the results of biochemical investigation of the Campbell visual cortex.

The retinotoxic drugs, two diaminophenoxy compounds and iodoacetate which are retinotoxic in other species at the dose schedule used, failed to cause any changes in the P.V.G. rat by ophthalmoscopic, histological or quantitative investigation. It would seem the rat eye is relatively resistant to such drugs, and it is suggested that species other than the rat should be used to determine retinotoxicity.

CONTENTS

		Page
CHAPTER I	- INTRODUCTION AND LITERATURE SURVEY	1
CHAPTER II	- QUANTITATION LITERATURE REVIEW	51
CHAPTER III	- MATERIALS AND METHODS	74
CHAPTER IV	- THE EYE	109
CHAPTER V	- THE VISUAL CORTEX	122
CHAPTER VI	- IDENTIFICATION OF CELLS . . .	154
CHAPTER VII	- MISCELLANEOUS PATHOLOGY . . .	171
CHAPTER VIII	- GENERAL DISCUSSION	183
CHAPTER IX	- BIBLIOGRAPHY	210

CHAPTER I

INTRODUCTION AND LITERATURE SURVEY

	Page
SECTION 1. CHOICE OF SPECIES	2
SECTION 2. THE VISUAL SYSTEM OF THE NORMAL RAT	3
(a) Composition and Function of the Visual System in the Adult	
(i) The Eye	
- Structure	3
- Function	6
(ii) The Pathways and Higher Centres	
- Optic Nerve	7
- Optic Tract	7
- Lateral Geniculate Body	8
- Retinohypothalamic Connection.	11
(iii) The Visual Cortex	
- Structure	12
(b) The Development of the Visual System	
(i) Prenatal Development	19
(ii) Postnatal Development	23
SECTION 3. DISEASES OF THE VISUAL SYSTEM OF THE RAT	
(a) Diseases caused by Agent Acting Antenatally	
(i) Genetic Inherited and Suspected Genetic Diseases	26
(ii) Experimental Non Inherited Disease due to Environmental Factors	30
(b) Diseases caused by Agent Acting Postnatally	
(i) Postnatal Expression of Hereditary Malformation	36
(ii) Non-Hereditary Disease..........	37
SECTION 4. SUMMARY	49

CHAPTER I.

SECTION 1. CHOICE OF SPECIES

This work was carried out, with the aim of deriving a quantitative method to assess damage to the visual system. The rat was chosen as the species for the work because it is the most commonly used laboratory animal, in addition to its standardisation, low cost and tractability. Although the rat is not an ideal subject ophthalmoscopy is possible, and was performed so that some assessment of the development of lesions in the live animals could be made.

Furthermore, rats with inherent reproducible pathological change in the visual system, the Campbell rat with retinal dystrophy and control rats, the Piebald Viral Glaxo (P.V.G.) strain which rarely, if ever, shows any spontaneous retinal degeneration, were available for comparison.

The Campbell strain originally described by Bourne et al (1938a, b) is a strain of pink eyed tan hooded rats. A failure in the final stages of development of the rod layer of the retina leads to complete blindness after the age of 3-4 weeks. This condition has a simple autosomal recessive inheritance (Dowling and Sidman, 1962). No pigment is present in the eye.

The sighted strain was originally derived from a stock of normal black hooded rats in the Glaxo Laboratories Limited with pigment present in the iris, pigment epithelium layer of the retina and choroid.

SECTION 2. THE VISUAL SYSTEM OF THE NORMAL RAT

(a) Composition and Function of the Visual System in the Adult Rat

The visual system of the rat consists of the eyes, optic nerves, optic chiasma, optic and accessory tracts and related higher centres, the lateral geniculate body, pretectal area, superior colliculus and visual cortex (Fig. 1, V.2).

i) The Eye - Structure

The eye of the rat is laterally directed, with a binocular field of about 117^{o} with maximum convergence, leaving a monocular temporal field of about 90^{o} on either side (Lashley, 1934a). Globular in shape, the eye consists of two modified spheres placed one in front of the other, namely the shallow anterior chamber and the larger posterior chamber, separated by the relatively large lens. The rat being a nocturnal animal, it has a large posteriorly positioned lens in contrast to the diurnal animals which have a small anterior lens (Duke Elder, 1958). The ratio for the weight of the eyes to the body weight is about 0.13% (Donaldson, 1924).

The cornea of the rat covers about 148^{o} of the circumference of the bulb (Lashley, 1932). It consists of epithelium of 6-8 layers of cells, a relatively thin Bowman's membrane, substantia propria of connective tissue fibres with a few interspaced nucleated cells, and a single layer of flattened cells forming Descemet's endothelium. Nerve endings in the rat cornea have been described (Zander and Weddel, 1951). The transparent cornea, which is avascular, is dependant mainly on the limbal vascular plexus for its nutrition by diffusion (Friedenwald et al, 1952). Macroscopically the rat cornea frequently contains irregularities and opacities which make ophthalmoscopic examination difficult (Fry, 1949). The iris extends as a thin highly vascular sheet, pigmented in hooded rats and non-pigmented in albinos, from the ciliary body to the region of the anterior surface of the lens. The sphincter muscle is strongly developed and there is no dilator muscle (Koganei, 1885). The diameter of the pupil varies from 0.5 mm up to 4.25 mm in the adult rat (Lashley, 1932).

The lens of the rat is nearly spherical, and occupies nearly two-thirds of the intraocular cavity (Fry, 1949) and weighs about one-third of the total weight of the eye (Donaldson, 1924). The lens is elastic and transparent and is held by a well developed suspensory ligament. A small rudimentary ciliary muscle is present which is continuous with Descemet's membrane anteriorly and with the inner layer of scleral fibres posteriorly (Lashley, 1932).

The outer coats at the back of the bulb comprise the sclera and choroid. The sclera is composed of coarse connective tissue fibres, blending anteriorly with the cornea. The optic nerve leaves the eye through the lamina cribosa, a foramen in the sclera (Friedenwald et al, 1952).

The choroid layer between the sclera and pigment layer of the retina is highly vascular, and pigmented in hooded rats, and consists of blood vessels and connective tissue. In albinos this layer is non-pigmented and much reduced in size (Lashley, 1932).

The retina of the rat covers about 175° of the circumference of the eye. The rat retina is similar in structure to that of man in that it consists of ten layers (Last, 1968). There are two main differences - the rat has a rod dominated retina (Duke Elder, 1958 - a ratio of rods to cones of 100:1) and the large number of rod nuclei form a thick prominent outer nuclear layer. Furthermore, in some strains pigment is absent from the pigment epithelium. The inner nuclear layer is considerably thinner, and the nuclei composing it are distinctly bigger than those of the outer nuclear layer. This reduction in cell number suggests there is a convergence of many visual cells on each bipolar or inner nuclear layer cell. The inner nuclear layer also contains horizontal cells as well as the nuclei of Müller's supporting fibres. A further reduction in cell number is evident in the ganglion cell layer (Tansley, 1961).

The eye of the adult rat is supplied by the ophthalmic artery which divides forming the central retinal artery and the ciliary artery. The central retinal artery supplies the retina by forming usually six symmetrically arranged retinal arteries radiating out from the optic disc, i.e. on the inner surface of the retina (Janes and Bounds, 1955). In addition a deep capillary network forms between the inner nuclear layer and the outer retinal layers (Cairns, 1959). The ciliary artery supplies the choroid and the iris. The central retinal vein drains the eye by means of four vorticost veins (Janes and Bounds, 1955).

The anatomy of the orbital glands in the rat has been described (Venable and Grafflin, 1940). The extrinsic muscles of the eye are the usual four rectal and two oblique (Lashley, 1932).

The Eye - Function

The eye performs two main functions - first as an optical instrument for collecting light waves from the environment and projecting them as images on to the retina and secondly as a sensory receptor which sends information to the visual areas of the brain (Johnson, 1966).

The cornea, lens, aqueous and vitreous humours serve to direct light rays on to the retina through which the rays pass to affect the photoreceptor processes, the rods and cones. In the rods exposure to light bleaches a reddish-purple pigment rhodopsin, and by a process not yet understood this causes excitation of the receptor.

Rhodopsin, a complex of proteins (opsin)phospholipids and the chromophore retinal, absorbs light and the 11 cis retinal changes to the all transform. The molecule then loses opsin and the retinal remaining is converted to retinol which is taken up by the pigment epithelium (Reading, 1970). The pigment epithelium, necessary for the regeneration of rhodopsin, also supports the outer segments of the rods and plays an important part in nutrition of the retina (Bernstein, 1961).

The impulse generated in the receptor cell passes via the bipolar cell to the ganglion cell layer and thence out of the eye to the higher centres. In a predominantly rod retina, with the reduction in cell numbers from the outer nuclear layer to inner nuclear and ganglion cell layers, there must be a high degree of summation, i.e. the impulses from a large number of rods will finally be concentrated on one ganglion cell, and this seems to be the basis of the high sensitivity of the rod retina (Tansley, 1961).

ii) <u>The Pathways and Related Higher Centres</u>

<u>Optic Nerve</u> The optic nerve is formed by fibres from the ganglion cells of the retina, converging at the optic papilla. The optic nerve pierces the choroid and sclera, and passes via the optic foramen into the cranial cavity where it partially decussates with its fellow and continues on as the optic tract (Zeman and Innes, 1963). 90% of the optic fibres in the rat cross at the chiasma and run in the contralateral optic tract (Polyak, 1941; Hayhow et al, 1960). The optic nerve is 7-8 mm. in length (Lashley, 1932).

Bruesch and Arey (1942) using a strip method of fibre estimation on sections stained with silver, found a difference in the total fibre number in the optic nerves of albino and pigmented rats, 74,800 as against 80,100 respectively. In addition, by exposing the optic nerve to osmium tetroxide and counting the myelinated fibres, they found no unmyelinated fibres in the grey rat, but 21% (15,500) unmyelinated in the albino.

Forrester and Peters (1967) however, using both light and electron microscopy on Araldite sections of optic nerve of albino rats, found a much higher number of fibres (117,000) and although 0.18% of these fibres had no myelin they assumed that these were cut at the node of Ranvier and that all the fibres were myelinated.

<u>Optic Tract</u> The optic tract runs dorso-caudally from the optic chiasma to the lateral geniculate body, pretectal area and superior colliculus. An accessory optic tract has been described (Hayhow et al, 1960) composed of inferior and superior fasiculi which

terminate in the dorsal and basal portions of the medial terminal nucleus of the accessory optic tract; some of the posterior fibres of the superior fasiculus terminate in the dorsal and lateral terminal nuclei of the accessory optic tract. The medial terminal nucleus is also known as the tractus peduncularis transversus nucleus (Bechterew, 1894), post optic tract nucleus (Bochenek, 1908), opticus tegmenti nucleus (Tsai, 1925) and nucleus of the basal optic root (Gillilan, 1941; Zeman and Innes, 1963).

The function of the optic nerve and optic tract is to relay impulses to the higher centres (Davson, 1972).

Lateral Geniculate Body The lateral geniculate body in the rat is an unlaminated curved structure, at the ventral end of the metathalamus, its surface covered by white matter. It is composed of two cellular divisions, a large dorsal and a smaller ventral part separated by a prominent fibre plexus. Neither of these divisions are homogeneous with respect to cell type. In the dorsal part there is a tendency for large cells to occur in the middle third, and the deeper regions rather than in the superficial third. The ventral part is further divided into medial and lateral portions; the medial region having no relationship with retinal fibres, the lateral having its largest cells in posterior region of the nucleus (Hayhow et al, 1960). The ventral lateral geniculate body fuses ventrally with the zona incerta (Fifkova and Hassler, 1969) forming the lateral terminal nucleus of the Accessory Optic Tract (Hayhow et al, 1960).

Both crossed and uncrossed fibres of the optic tract terminate in both the dorsal and ventral parts of the lateral geniculate body, with a tendency to segregation, establishing a primitive laminar pattern (Hayhow et al, 1962). Fifkova and Hassler (1969) found a difference between the rostral and caudal portions of the lateral geniculate body, the rostral end containing mainly large neurones with large thickly branching dendrites whereas the caudal end comprised mainly small neurones.

The dorsal part of the lateral geniculate body projects fibres via the optic radiation to the striate cortex (Lashley, 1934b) to the peduncle of the superior colliculus, and surrounding areas (Ariens Kappers, 1936-65).

The ventral part of the lateral geniculate body is connected to the superior colliculus via the peduncle, and forms an important efferent path to the ventral nucleus from the tectum. The cells of the ventral part of the lateral geniculate body are not, it is suggested, dependent upon retinal stimulation for their integrity (Tsang, 1937; Hayhow, 1958; Hayhow et al, 1962). The function of the lateral geniculate body is to act as an essential centre (Ariens Kappers, 1936-65) to make possible a fusion of impulses from corresponding parts of two retinae (Brodal, 1969).

The anterior choroidal artery and the posterior cerebral artery form the blood supply of the lateral geniculate body (Tsang, 1935).

The pretectal area has been described (Bucher and Nauta, 1954 as being composed of

(a) a superficial group of nuclei related to the brachium of the superior colliculus, the medial and lateral portions of the nucleus of the optic tract.

(b) more deeply lying nuclei, the pretectal nucleus, the deep pretectal nucleus and the medial pretectal area.

These authors emphasise that the middle and deep grey layers of the colliculus extend into the pretectal region over a considerable distance and fuse with most of the cell groups of the latter. It has been demonstrated that the pretectal region is an optic terminal centre ennervated by both crossed and uncrossed optic axons, but predominantly the former. The principal terminal centres are the lateral nucleus of the optic tract and the pretectal nucleus (Hayhow et al, 1962).

The decrease in size of the superior colliculus phylogenetically is to a considerable extent in inverse proportion to the development of the dorsal lateral geniculate nucleus since the latter assumes many of the functions carried out by the superior colliculus (Ariens Kappers, 1936-65).

The superior colliculus in the rat is composed of lamina as follows :

(1) Stratum zonale
(2) Superficial grey layer (stratum griseum)
(3) Stratum opticum
(4) Middle grey layer
(5) Middle white layer
(6) Deep grey layer
(7) Deep white layer (Ariens Kappers, 1936-65).

Other authors tend to name the layers somewhat differently (Tsang, 1937; Hayhow et al, 1962).

The contralateral optic tract afferent to the superior colliculus terminates in the superficial stratum opticum and the adjacent superficial stratum griseum (Hayhow et al, 1962). The ipsilateral optic afferents are somewhat sparse and terminate in the stratum zonale and superficial stratum griseum (Bodian, 1937; Nauta and van Straaten, 1947).

Retinohypothalamic Connection Light influences a large number of autonomic functions in most mammals, for example hypothalamic control of gonadotrophin secretion, and the possibility is generally accepted that this influence must be mediated by some nervous connection between the retina and the hypothalamus. (Butler and Donovan, 1971; Sousa-Pinto and Castro Correia, 1970). There have been many conflicting reports on this connection, with failure to find this pathway in the rat (Nauta and van Straaten, 1947; Hayhow et al, 1960; Kiernan, 1967) which seems to be due to the type of silver stain used, and the interpretation of the resulting "terminal degeneration" impregnation. It is important to be aware that the various silver impregnations differ with respect to the impregnation of degenerating fibres and terminals, so that negative findings may not be decisive. Similarly pseudo-degenerations can occur and the final proof can only be obtained from electron microscope studies (Brodal, 1969).

However, by using the Fink Heimer method Sousa-Pinto and Castro-Correia (1970) demonstrated a pathway to the preoptic, arcuate and ventromedial hypothalamic nuclei in the rat, and confirmed this finding by electron microscopy, showing degenerating boutons in the arcuate nucleus (Sousa-Pinto, 1970).

Further support for this pathway has been produced by Moore et al (1967) who measured the activity of hydroxyindole O-methyl-transferase (H.I.O.M.T.) an enzyme in the pineal gland, the activity of which is controlled by environmental lighting. Orbital ennucleation, and accessory optic tract transection suppressed the effects of light on this enzyme, whereas destruction of the primary optic tract and superior accessory tract had no effect.

In addition by using autoradiography a possible pathway from the eye to the suprachiasmatic nucleus of the medial hypothalamus has been described (Moore and Lenn, 1972), again confirmed by electron microscopy.

It would appear that the normal silver stains for terminal degeneration are not reliable enough for these fine accessory tracts and the use of electron microscopy would seem the most obvious solution.

iii) The Visual Cortex - Structure

The visual cortex, also known as the striate area and Area 17, is found on the dorsal convexity of the occipital pole. The extent of the striate area described by the various authors varies - the early maps using cytoarchitectural criteria of Fortuyn (1914), Sugita (1917), Volkman (1926) and the later generally used map of Kreig (1946a) show a more extensive Area 17 than the maps of Waller (1934) and Lashley (1934b) who used retrograde degeneration studies. Again with physiological criteria, Area 17 of Adams and Forrester (1958) is much less extensive than Le Messurier's (1948) who also used physiological techniques. Cytoarchitectural techniques have been criticised (Lashley and Clark, 1946; Clark, 1962) but for the purposes of this work the generally accepted map of Kreig (Nauta and Bucher, 1954; Lund, 1966) has been used, restricting the visual cortex to the primary visual area, Area 17, and not extending beyond the borders into Area 18 and 18a.

Area 17 is described as a large pyriform field forming an extensive part of the posterior third of the dorsal aspect of the hemisphere with a uniform structure and a sharp border separating it from Area 18 (Kreig, 1946a).

The cerebral cortex of the rat consists of six lamina, from the pial surface towards the white matter these are named:

(1) Lamina zonalis, molecular or plexiform layer	
(2) Lamina granularis externa, external or outer granular layer	Supragranular L. 2 and 3
(3) Lamina pyramidalis or medium sized pyramidal layer or external pyramidal layer	
(4) Lamina granularis interna or internal or inner granular layer	
(5) Lamina ganglionaris or large pyramidal or lateral pyramidal or ganglionic layer	Infragranular L. 5 and 6
(6) Lamina multiformis or polyhedral layer or layer of fusiform cells	

(Truex and Carpenter, 1969; Zeman and Innes, 1963).

The supragranular layers are more extensive, the latest to arise, and the most highly differentiated in man. In mammals other than man the infragranular layers are well developed and are connected directly with subcortical structures by descending projection systems (Truex and Carpenter, 1969).

Area 17 in the rat does not have the highly stratified development found in the striate area of higher mammals. The lamination has been described by Kreig (1946b) as follows:

L_1 = Relatively acellular zone and clearly visible.

$L_2 - L_3$ = Merged as a single layer, composed of small pyramidal cells, many of which are polyhedral and almost granulous (sic) with a small apical process visible.

L_4 = Distinct and composed of dark-staining, very closely packed small granule cells.

L_5 = Thin with an open structure, containing broad pyramidal cells which also stain darkly and send basal processes in all directions of the tangential plane. There are other smaller less well staining pyramidal cells present.

L_6 = Thick, with two types of cells present - flattened large granular cells and scattered larger bun-shaped cells, which have a tendency to form horizontal strata.

Layers 2 and 3 which are merged as a single layer are equal in thickness to layer 4, and layers 2, 3 and 4 are equal in thickness to layers 5 and 6.

The work of Rose (1912; 1929) is quoted by Tsang (1937) who describes the cortex as follows:-

L_{2-3} = Narrow, compared with surrounding areas, consisting of small, round pyramidal cells.

L_4 = Exceptionally wide, with densely packed granular elements.

L_5 = Divided into 3 sublayers, with the outer and inner poor in cells but the central sublayer a conspicuous layer of large pyramids.

L_6 = Narrow, but rich in cells.

Although the original work by Rose was on mice, the findings do apply to the rat and a comparison with Kreig's work shows how authors have disagreed over the lamina divisions and sublayers. As Clark (1962) wrote "what can be the magnitude of large, medium and small? When does many become numerous" and exceptionally wide become thick?

Lund (1966) suggests that differences may arise from different strains of rats, but the principal cause of variation is the poor criteria available for defining the area. The centre of the area, however, is distinct with a thick Layer 4 composed of many densely packed cells and this is the characteristic of the area, graduating towards the periphery into the pattern of the neighbouring areas.

With few exceptions, the nerve fibres of the neocortex form a three dimensional network, the neopallial white matter. In addition, three types of loosely arranged bundles can be distinguished which extend through the cortex parallel to the surface - association, commissural and projection systems. Association systems connect neocortical areas in the same hemisphere, commissural connect the two opposite cortices and projection systems connect the cortex with subcortical structures (Zeman and Innes, 1963).

The striate area of the cat has been analysed (O'Leary, 1941) in detail. There has not been any similar detailed investigation in the rat, although an examination using silver impregnation methods of all the cortical areas in the albino rat has been reported (Vaz Ferreira, 1951). This work showed a difference within Area 17, namely that the density of fibres were greatest in the anterior part. This was to be expected as Solnitzky and Harman (1946) had found structural differences between the peripheral and central sectors of the cortical visual area in primates.

Vaz Ferreira (1951) found on using a silver stain :

L_1 - contains sublayers 1a superior tangential layer
1b tangential layer

L_{2-3} - the suprastriate area which is fibre poor, but with some radial fibres present. The density, however, increases with depth and merges with the external stria of Baillarger.

L_4 - The external stria of Baillarger, although not well developed in the rat

L_{5-5a} - The intrastriate area which contains radial fibres and a thin additional stria, probably the rudiment of the double stria of Baillarger of higher forms.

5b - Merged with L_6.

L_6 - Uniform structure, formed by horizontal and radial fibres, known as the infrastriate area.

The external stria of Baillarger narrows towards the medial aspect of the area, as does layer 5a. The general stratification also tends to become more blunted close to the medial edge of the area (Vaz Ferreira, 1951).

Area 17 has the fewest myelinated fibres of all the sensory receptive areas. A radiata is present and extends through L_4 although quite small. A uniform stratum of tangentially running fibres is present in layers 5 and 6, which tend to diminish medially

(Kreig, 1946b). From this it can be seen that there is general agreement between these authors on the fibres found in the visual cortex.

By means of retrograde degeneration studies after damage to the area striata Lashley (1934b) concluded that the optic afferents of the visual cortex came from the dorsal nucleus of the lateral geniculate body.

This projection of the dorsal lateral geniculate nucleus on the cortex shows a high degree of localisation which must approach a point to point correspondence to give the high differentiation obtained. The most anteromedial part of the striate areas represents the dorsal meridian of the eye, the lateral margin successive sectors of the temporal quadrants and the nasal quadrants are projected on the medial border of the visual cortex (Lashley, 1934b).

In the rat each point of the striate area has homolateral associations with all remaining parts of Area 17, with Area 18 and a medial part of 18a, mainly established by tangential association fibres (Nauta and Bucher, 1954).

Commissural fibres appear to originate in all parts of the striate cortex and pass to the lateral 2/3rds of the contralateral striate area and an adjoining strip of Area 18a. The medial 1/3rd of 17 and Area 18 do not receive association fibres from the contralateral Area 18.

Thus the afferent supply to the rat visual cortex will consist of the geniculostriate projection (thalamocortical) and association fibres from 18, 18a and other parts of 17. The lateral 2/3rds of Area 17 will also receive commissural fibres from the contralateral

17, 18 and 18a. These afferents end mainly in lamina 6, although some terminate in L_4 and L_5 and a very few reach L_2 and 3.

A pathway arising within the visual cortex and terminating in the superior colliculus has been described (Nauta and Bucher, 1954; Lund, 1966). The visual area as defined by Lashley (1934b) gives rise to a corticotectal pathway which terminates principally within the three most superficial lamina of the superior colliculus, whereas corticotectal pathways anterior and medial to this visual area project only to the stratum album and stratum griseum (Lund, 1966).

In addition to the superior colliculus the striate cortex has connections with the lateral geniculate nucleus (mainly the ventral part), the nucleus lateralis posterior thalami, pretectal region, zona incerta and pons (Nauta and Bucher, 1954).

This review on the literature of the visual system of the rat agrees with Ebbesson (1970) in that it reveals areas of emphasis and areas of neglect. Little is known about the projections of the ventral nucleus of the lateral geniculate body, the pretectal nuclei and the nuclei of the accessory optic system in any species.

The basic projections of the retina appear to be :

(1) Hypothalamus

(2) Nuclei of accessory optic tract

(3) Dorsal thalamus

(4) Ventral thalamus

(5) Pretectal region

(6) Optic tectum.

From the dorsal nucleus of the lateral geniculate body the geniculostriate tract passes to the visual cortex, which in turn sends afferents to many of the subcortical centres.

(b) The Development of the Visual System

i) Pre-Natal Development (Fig.34, V.2)

After copulation and fertilisation, the fertilised rat ovum divides, reaching the fourth cleavage stage 96 hours after ovulation. Implantation and uterine decidual reaction begins at 5-6 days, with completion of decidua 6-7 days after copulation. 8-9 days after copulation the differentiated germ layers, the mesoderm, ectoderm and endoderm begin to develop (Nicholas, 1949).

The central nervous system of mammalian embryos can be recognized as a long slipper-like area, known as the neural plate. This neuroectoderm becomes elevated forming the neural folds, the depressed centre produced is called the neural groove. Further development results in the neural tube with neural crests (Langman, 1968).

By a process of unequal growth producing a series of flexures the anterior end of the neural tube forms swellings or vesicles separated by constrictions. These vesicles can be recognised as the forebrain, midbrain and hindbrain. The forebrain undergoes many changes, the posterior portion gives rise to thickenings which become the thalamus, hypothalamus and subthalamus, and the anterior portion forms two thickened bulges which constitute the cerebral cortices (Ham, 1965).

By day 11 a lateral outgrowth from the forebrain or prosencephalon vesicle forms the optic vesicle which becomes separated from the forebrain by a pedicle, the optic stalk. This neural ectoderm of the optic vesicle becomes invaginated and forms the two layered optic cup. The surface ectoderm distal to the optic vesicle thickens to form the lens vesicle on day 12,which eventually fills in and becomes the lens. Mesoderm grows round the lens and optic cup forming the cornea, and also condenses around the optic cup forming choroid and sclera, first visible on day 15-17 of gestation. Ectoderm forms the epithelium of the eyelids, cornea and glands of the lids and the lacrimal glands. (Last, 1968; Mann, 1949; Ham, 1965; Berson, 1965).

The retinal primordium, the pars optica retinae consists at first of a single layer of columnar epithelium, which divides and forms layers. Mitosis occurs at the side next to the future pigment layer so that the oldest cells get pushed towards the future vitreous. The cells differentiate and form the specialised layers (Last, 1968), and this process is still in progress when the rat is born (Detwiler, 1932).

The classical theory of His (1889) divides the neural tube into three zones, the ependymal zone bordering the lumen of the tube, a middle mantle zone and the outermost or marginal zone. The ependymal zone consists of high columnar epithelial cells and large round or germinal cells, which His believed gave rise to spongioblasts and neuroblasts respectively. The neuroblasts and spongioblasts were believed to migrate into the densely packed nuclear zone, the mantle zone, after their formation. The outermost zone, the marginal zone was thought to consist of the peripheral processes and axons of the neuroblasts. Langman (1968) and Hardesty (1904) agreed with these concepts, although they preferred to think of all the cells in the neural wall as a syncytium.

Despite general acceptance of these theories in modern textbooks (Truex and Carpenter, 1969; Ham, 1965) criticism has been raised notably by Sauer (1935a, 1935b; 1936, 1937) who concluded that the cells in the ependymal and mantle layers were not of two different types, but of essentially similar cells, extending from the lumen of the tube to the external limiting membrane, and attached to each other at the lumen by terminal bars. The suggestion made was that when a cell prepares for mitosis, the nucleus moves gradually towards the lumen and the cell contracts, using its terminal bars as an anchor, thus forming a round cell shape.

This theory of Sauer has been confirmed by work using colchinine (Watterson et al, 1956),microspectrophotometric DNA measurements (Sauer and Chittenden, 1959), autoradiography (Sauer and Walker, 1959; Langman and Welch, 1967), and electron microscopic techniques (Duncan, 1957).

The neuroblast is first visible on the periphery of the neural tube, as a cell with small processes, characterised by paired membranes of endoplasmic reticulum (Bellairs, 1959), and later by their typical cytoplasmic structures. The neuroblasts are attached initially to the lumen by a "transient dendrite" but this disappears as the neuroblast migrates (Barron, 1946). Primitive neuroblasts are described (Langman et al, 1966) as having a large round nucleus, pale nucleoplasm and a dark staining nucleolus.

To explain the formation of various layers of the cerebral cortex it was suggested that the neuroblasts migrate to the pial surface in three successive waves, with the latest formed cells pushing the previously formed neuroblasts ahead of them (Tilney, 1933). However, using autoradiography it was found that neuroblasts formed later migrate through previously formed layers (Angevine and Sidman, 1961) and in fact may even migrate subsequently in retrograde fashion to take up a deeper position (Berry and Rogers, 1965). The migration pattern through the previously formed layers has been found in the rat (Hicks et al, 1961; Berry and Eayrs, 1963; Langman and Welch, 1967) but not the retrograde migration.

The development of glial cells has been studied in the mouse (Smart and Leblond, 1961). Having identified the nuclear characteristics of glia cells, they found additional cells as well as the oligodendroglia, astrocytes and microglia. These extra cells, the small dark and medium dark nucleus cells found in the subependymal layer, are primitive spongioblasts and give rise to oligodendroglia and astrocytes (Smart, 1961). The origin of microglia is not clear. Konigsmark and Sidman (1963) labelled mouse blood leucocytes with ^{3}H thymidine, which is incorporated into DNA and is distributed equally between daughter cells. No labelled leucocytes appeared in the brains of normal rats, but if stab wounds were made in brains of these mice then labelled macrophages could be recorded in these wounds, thus suggesting that the circulating leucocytes are a major source of brain macrophages. Labelled cells also appeared along the small vessels in endothelial and adventitial cells, which were indistinguishable from the activated microglial cells of del Rio-Hortega.

Other work revealed that, following heavy particle irradiation of mouse brain, many phagocytes appeared to be derived from pericytes and not from any glial element within the brain parenchyma (Maxwell and Kruger, 1965).

Electron microscope studies have as yet failed to clarify the situation - "if gitter cells are derived from leucocytes and other mesodermal elements such as pericytes, what are the microglial cells of del Rio-Hortega ?" (Maxwell and Kruger, 1965).

ii) Post-Natal Development

The rat, like other rodents, is born blind and begins to develop vision at 9-10 days of age (Dowling and Sidman, 1962). The development of the retina from birth to maturity has been described (Detwiler, 1932; Tansley, 1933-34). On day 1 there is no separation of the inner and outer nuclear layers, which exist as a broad band of nuclei along the choroidal border. In the first five days of life there is much mitotic activity along this choroidal border, until between 5-10 days the inner and outer nuclear layers separate (Lucas et al, 1966); mitotic figures being sparse after the sixth day (Detwiler, 1932). The inner segments of the rod nuclei of the outer nuclear layer begin to extend past the external limiting membrane at 9 days; the outer segments are visible by 10 days. At 10 days electrical activity can first be recorded in the cat retina (Dowling and Sidman, 1962). The rods grow rapidly from this stage; by 17-18 days differentiation of the retina is nearly complete although the rhodopsin content at this stage is only about 50% of the adult level. After 18 days of age, the outer segments continue to lengthen until they reach the adult length by about 39 days of age. The rhodopsin content likewise increases to the adult level (Dowling and Sidman, 1962).

The rat eye at birth is dependent on the hyaloid vessels which occupy most of the vitreous space and cover the surface of the retina. The hyaloid vessels persist till the 7th day, by which time the retinal vessels have almost reached the periphery of the retina. The hyaloid vessels rapidly involute, becoming vestigial by day 11. The retinal circulation is adult in form by the 15th day (Cairns, 1959; Agrawal, 1965; Engerman and Meyer, 1960).

The quantitative aspects of growth of the lens, eyeball and cornea of the rat have been described (Norrby, 1958).

The rat cerebral cortex at birth is not mature, in fact local differentiation has barely commenced (Eayrs and Goodhead, 1959).

The cortex at birth is made up of undifferentiated cells, tightly packed into vertical columns between which relatively extensive extracellular spaces, developing processes and blood vessels can be found (Caley and Maxwell, 1968a).

By measuring the thickness of the cerebral cortex, a rapid period of growth was found from birth to 10 days with cell multiplication and growth and a slower increase in thickness from 10-20 days due mainly to enlargement of cell bodies and growth of dendrites and axons. At 20 days the cerebral cortex had almost reached maximum thickness, with a slight increase up to 90 days due to ingrowing axons and myelination (Sugita, 1918).

However, studies of the development of area 2 (Kreig,1946a,b) suggested that the period from birth to 6 days is the period of the most rapid change in cell/grey matter coefficient (relationship between the mass of the grey matter to that of the perikarya of its constituent neurones),whereas the density of axons increases maximally 6-18 days and that of dendrites 18-24 days (Eayrs and Goodhead,1959).

In his description of the development of the cerebral cortex of the rat, Tilney (1933) found that cortical stratification occurs day 1-5, and the optic tract showed a considerable increase in small dark cells. By 10 days after birth myelin is apparent in many tracts. From 10-20 further myelinisation occurs and myelinated fibres can be traced from the optic tract to the tectum. A general increase in cortical thickness has taken place, and it appears that the cerebral cortex has assumed predominance in behaviour regulation.From 20 days to adulthood further specialisation of cells occurs and myelinated intercortical fibres appear.

Electron mi croscopic studies of the developing rat cerebral cortex suggest that the undifferentiated cells at birth differentiate into either neuroblasts or spongioblasts. In the case of neurones this maturation occurs in a gradient from the innermost layers towards the surface. By the end of 21 days the cortical tissue and its neurones are apparently mature and the adult pattern of cortical fine structure is established (Caley and Maxwell, 1968a, b).

The development of the cerebral cortex can thus be summarised as an intrauterine development of strata by a process of cell migration, with the oldest cells innermost. After birth these indifferent cells differentiate into either neuroblasts or spongioblasts and establish the adult pattern by 21-24 days.

SECTION 3. DISEASES OF THE VISUAL SYSTEM OF THE RAT

(a) Diseases Caused by Agent acting Antenatally

Although the rat visual system is not mature at birth, it was decided to separate conditions and agents which affect the visual system into antenatal and postnatal conditions. Agents acting antenatally will produce malformations whereas those acting postnatally will result in loss of function with degeneration or death of cells.

An overlap occurs in this classification due to abiotrophies. Abiotrophies are hereditary conditions that become manifest at some period of postnatal life, the tissue having functioned apparently normally until then (Sorsby, 1963). It is more descriptively termed a heredo-degenerative condition. The classical example of an abiotrophy is retinitis pigmentosa in the human.

i) Spontaneous Genetic (Inherited) and Suspected Genetic Diseases

Disease occurring in intrauterine life (congenital disease) may be due to an abnormality of the genotype-an hereditary disease, or environmental factors affecting a foetus with a normal genetic composition. There are many reports of congenital defects of the eyes in rats and mice, mainly on experimentally produced malformations. Some reports on naturally-occurring hereditary conditions have been published, however. Hereditary congenital defects occur in the population in proportions explicable by the laws of inheritance. They may be obvious at birth, or as in the abiotrophies become manifest at some period in postnatal life. Some disorders such as the hereditary tumour retinoblastoma in the human, may or may not be present at birth since the size of the tumour at birth may be so small as to escape detection.

The diseases will be treated in the following order:

(α) Cataract

(β) Hereditary cataract and retinal degeneration

(γ) Microphthalmia and anophthalmia

(δ) Eye and tooth abnormalities

(ε) Colobomata

(ζ) Congenital hypertrophy.

(α) <u>Cataract</u> visible first at 4 days and affecting both eyes was found to be due to a simple, dominant autosomal gene in the albino rat (Smith and Barrentine, 1943). Cataract and skull malformations in the Sprague-Dawley rat due to a simple autosomal recessive have also been reported (Smith et al, 1969).

(β) <u>Hereditary cataract and retinal degeneration</u> in rats was first reported in 1938 (Bourne et al, 1938a, b). From these original rats the strain known as "Campbells" was bred. At birth these rats were thought to be normal, and the disease, due to retinal changes, became apparent at 21 days. As it was thought the rat retina was mature at 17 days (Bourne et al, 1938b) and the degeneration was not present until 21 days, this condition was considered an abiotrophy.

Histologically the first stage was death of the rod nuclei in the outer nuclear layer, followed by complete degeneration of the layer and disintegration of the rods over the following 28 days. By 7 weeks the degenerate rod and outer nuclear layers were replaced by a glial network and alterations in the pigment layer. The retina as a whole convoluted at 11 months, and by 2 years of age the layered structure of the retina was lost (Bourne et al, 1938b).

The genetics of this condition were investigated (Bourne and Gruneberg, 1939) and found to be due to a simple autosomal recessive. Further investigation using chemical, physiological and electron microscope techniques revealed that the first abnormality recognised was the appearance of swirling sheets or bundles of extracellular lamellae between normally developing retinal rods and pigment epithelium at 12 days. Rhodopsin content was twice the normal value and a somewhat limited investigation of this rhodopsin suggested it was normal. Photoreceptors attained virtually adult form and electroretinogram function at 21 days and then rod inner segments began degenerating, with loss of electroretinogram sensitivity and depression of the a waves. Outer segments followed with decrease in rhodopsin, eventually leading to loss of photoreceptor cells and the b wave of the electroretinogram (Dowling and Sidman, 1962).

Another investigation (Lucas et al, 1955) suggested that the degeneration started at 15-16 days after birth when amorphous masses of eosinophilic debris are visible in the distal segments of the rods. Furthermore, these authors suggest the rat retina is not completely differentiated until 5 or 6 weeks after birth, and thus the condition is not an abiotrophy.

The biochemistry of this retinal dystrophy has also been studied, and the most important findings are firstly an apparent overproduction of visual pigment (rhodopsin) in the rods of dystrophic rats. Secondly, there is an excessive local concentration of retinol (Vitamin A alcohol) in the "pigment layers" (the choroid, sclera and retinal epithelium after separation of the retinal layers); N.B. the shear line is at the level of the rods. This change occurs before the onset of histological changes. Finally the increase in retinol is retarded by dark rearing, and these observations suggest an overproduction of an unusually light labile rhodopsin, which with light causes the excessive local concentration of retinol in the pigment epithelium. This build up of retinol tends to cause breakdown of the membranes of lysosomes, thus releasing bound acid hydrolases and proteases, accounting for the cellular breakdown and disappearance of the cellular debris histologically (Reading, 1970).

Biochemical investigations of the visual cortices of normal and retinal dystrophic rats have shown a significant lowering of the RNA:DNA ratio, a reduced rate of RNA synthesis with labelled orotic acid and a lower rate of RNA polymerase activity in the blind rats. No difference was found in poly c synthetase activity. These results suggest a less active visual cortex in the blind animals possibly due to differences in the level of nervous activity (Dewar and Reading, 1970; Dewar and Reading, 1972).

Retinal dystrophy has also been reported in the mouse (Tansley, 1954, 1951) and in some breeds of dog (Barnett, 1966).

(γ) Microphthalmia and anophthalmia of a hereditary nature, although the mode of inheritance is unknown, was found in the white rat along with faulty development of optic nerve and retinal tissue (Browman, 1954; Browman, 1961). Hereditary microphthalmia, with degeneration of the retina in albino rats of polygenic origin has also been described (Campbell, 1943).

(δ) Eye and tooth abnormalities, which were not described in albino rats were reported (Jones, 1925). This condition may be hereditary although some experimental procedures were performed on the pregnant dam.

(ε) Colobomata of the optic nerve sheath with ectasias of the retina passing between the edge of the lamina cribosa and sclera was found in rats with congenital retinal degeneration. The colobomata showed a hereditary tendency, but the exact nature of inheritance has not been established (Nicholls and Tansley, 1938).

(ζ) Congenital hypertrophy of the eye, due to impaired drainage of aqueous humour has been described. This rare condition in the rat resulted from persistence of the pupillary membrane, incomplete development of the iris and ciliary body and involvement of the tissues at the angle of the anterior chamber of the eye (Addison and How, 1926).

ii) Experimental Non-Inherited Disease due to Environmental Factors

Most teratogenic agents do not produce one type of malformation only, i.e. they are non-specific. The type of malformation produced depends on the time of action in relation to the stage of foetal development (Malformation Timetable, Figs. 35-37, V.2), and on the strength of the agent operating. Hence one agent can produce more than one type of malformation.

The factors will be treated in the following order :

(α) Ionizing irradiation
(β) Hypothermia
(γ) Hypoxia and hypobarometry
(δ) Mechanical (surgical) damage
(ε) Stress
(ζ) Chemical
(η) Nutrition
(θ) Infections

(α) Ionizing irradiation - the primitive differentiating cells of the nervous system are probably the mammalian cells most easily destroyed by ionizing irradiation (Hicks, 1954). The optic primordium is very vulnerable to X irradiation - 25 rads are enough to disturb ocular development on the 9th day of gestation in the rat, whereas the teratogenous amounts for other organs are 100 rads or more (Wilson et al, 1953). The time of irradiation is also important, if early in pregnancy before the eye is formed, X-rays result in total absence or reduction of development, i.e. anophthalmia or microphthalmia and various other anomalies such as encephalocoele and persistence of the choroid fissure (Hicks, 1953; Rugh, 1958; Rugh and Clugston, 1954; Wilson et al, 1959). (Fig.35, V.2)

Later in pregnancy after the eyes have formed, a higher dose of radiation on the 13th day of gestation results in massive destruction of retinal cells within four hours. However, within a further 72 hours, these retinal cells have been phagocytosed and the retina repaired, the offspring having only a slight microphthalmia (Rugh and Wolff, 1955).

Ionizing radiations at low doses selectively damage the primitive neuroblasts and spongioblasts in embryo, foetal and young animals. The resulting malformations range from anencephaly through hydrocephalus to various kinds of microcephaly and cerebellar defects. When the dose of radiation remains constant the pattern of malformation is determined by the time during development at which the animal is irradiated (Hicks, 1954; Berry and Eayrs, 1963).

(β) Hypothermia - Lowering the body temperature of hamsters to below $0^{o}C$ for 45 mins. between day 1½ to day 8½ caused malformations, among which were bilateral or unilateral anophthalmia (Smith, 1957).

(γ) Hypoxia and hypobarometry - Eye abnormalities such as puckering of the retina and lens degeneration were found in rats subjected to low pressure of air (350-460 mm of mercury) on day 1-8 of gestation (Werthermann and Reiniger, 1950). The offspring of pregnant mice, submitted to low air pressure of 260-280 mm of mercury on day 16-17 of gestation, had lack of fusion of eyelids (open eyes), coloboma and eversion of the retina with absence of the chambers in varying degrees. Extraocular vascular haemartomas and hydrocephalus were also observed as were retrogressive changes within the lens and proliferation of vasoformative tissue (Ingalls et al, 1952).

(δ) Mechanical (surgical) damage - Various congenital defects were found in the offspring of hypothyroid rats, obtained by partial thyroidectomy 7-43 days before pregnancy. A high percentage of eye defects involving the lens with retinal folds, coloboma and anophthalmia was observed in embryos examined at 20 days of gestation (Langman and Van Faassen, 1955).

Surgical removal of the eye in foetal guinea pigs, between day 35-55 of gestation with a survival time of 1-9 days caused marked changes in the visual system. The optic nerve was degenerate and lacked intact fibres. Only partial decussation occurred at the chiasma, and the fibre content of the contralateral visual cortex and lateral geniculate body was reduced. The superior colliculus was particularly affected with virtual absence of the stratum opticum and collapse of the superficial grey layer (Hess, 1958).

(ϵ) Stress - the progeny of pregnant rats, subjected to various types of stress during gestation, showed after 22 days of age a decreased nucleic acid and protein concentration in hypothalamus, cerebral cortex and cerebellum. It was concluded that alterations in the humoral status of the dam are capable of inducing changes in the foetus that are reflected in aberrant neurochemical change during life (Petropoulos et al, 1972).

(z) <u>Chemical</u> - Trypan blue injected subcutaneously between day 5-7 of gestation produced congenital defects of the eye and nervous system. The eye defects ranged from anophthalmia to vacuolation and enlargement of the lens with retinal folding. Spina bifida and hydrocephalus were also produced. Eye defects could not be produced after emergence of the optic cup, i.e. after 14 days of gestation (Gillman et al, 1948; Gilbert and Gillman, 1954) (Fig.36,V.2)

Other investigators have likewise observed microphthalmia, anophthalmia and exophthalmia after injections of 1% trypan blue on day 7-9 of gestation (Tuchmann-Duplessis and Mercier-Parot, 1956).

The hypoglycaemic sulfonamides, carbutamide B255 and tolbutamide, and Actinomycin C, caused ocular malformations depending on the dose, the strain of rats and the age of the foetus. In the majority of cases the anomalies were considerable, whether single or multiple; some concerned the eye with a predominance of anophthalmia with colobomas, abnormal orientation of the primordia, retinal wrinkling and lens deteriorations (Tuchman-Duplessis and Mercier-Parot, 1958, 1959).

X-methylfolic acid on the 8th and 9th day of gestation in the rat causes colobomas, cystic retina with cysts, lenticular cysts, microphthalmia, anophthalmia and Morgagnian type cataracts (Nelson et al, 1955).

(η) Nutrition - Vitamin A deficiency in the pregnant rat produces congenital eye defects in the offspring. Colobomas, vitreous body fibrosis, retinal puckering and eversion, and rudimentary development of the iris and ocular chambers have been reported (Warkeny and Schraffenberger, 1946). An excess of Vitamin A can also prove teratogenous, with brain extrusion and gross eye defects (Cohlan, 1953). Other workers have reported anophthalmia, microphthalmia, colobomas, fibrosis of the vitreous body, retinal folding and intra-ocular haemorrhages in the offspring of pregnant rats and rabbits receiving from 35,000 to 1,000,000 I.U. of Vitamin A (Giroud and Martinet, 1959) (Fig. 37, V.2) Anencephaly due to a failure of closure of the neural tube has been produced in rat embryos. The dams of these embryos received 30,000 U.S.P. units of Vitamin A on day 8, 9 and 10 of pregnancy (Langman and Welch, 1966). Maternal deficiency of pantothenic acid produced anophthalmia and microphthalmia in the offspring (Lefebreves, 1951). Maternal folic acid deficiency often results in complex malformations of the eye, colobomas and retinal folding in the embryos (Giroud et al, 1954). Maternal folic acid deficiency in the rat also results in failure of closure of the neural tube (Nelson, 1960). Pike (1951) reported that rats on a diet deficient in tryptophan produced offspring with cataract. Multiple congenital abnormalities in rat embryos, including some ocular anomalies, have been produced by a riboflavine deficiency in the dam induced by galactoflavine (Nelson et al, 1956). The sugar galactose, when fed to pregnant rats at a level of 25% of the diet, produces cataractous changes in the lenses of the embryos (Bannon et al, 1945).

Thyroxine given to the dam has produced cataracts in the lenses of the offspring (Giroud and de Rothschild, 1951). So far malformations of the eye have not been reported after administration of antithyroid substances although maternal thyroidectomy does produce congenital ocular malformations (Langman and Van Faassen, 1955).

Disturbances of hydrocarbon metabolism, in particular diabetes, have often been thought responsible for embryopathies which are said to be three times more frequent in patients with these conditions than among normal women (Hoet et al, 1959).

(9) Infections - Rubella in the human, causing single or bilateral cataracts with atrophic appearance of the iris is widely known (Gregg, 1942). Toxoplasma in the human causes microphthalmia, persistent papillary membrane and chorioretinitis (Bamatter, 1947) although infecting rats with toxoplasma only produced cataracts (Giroud et al, 1954).

In summary, the sensitivity of the optic primordium to damage is shown by the number and range of agents which are capable of arresting its development. The result of damage to the optic primordium, i.e. the anomaly produced tends to be most serious if the agents act early in development, as is seen with hypervitaminosis A. Excess Vitamin A from day 5 - 8 of pregnancy produces anophthalmia, from day 8 - 11 anophthalmia and microphthalmia, from day 11 - 14 aplasias of the eyelids and only cataract from day 18 - 24 (Giroud and Martinet, 1956). Another essential factor is the genotype of the animal which influences the reaction to the teratogenous agent, and can result in considerable variation in the degree and type of malformation

(Tuchman-Duplessis and Mercier-Parot, 1961). An example of this is seen in galactose induced cataracts where the degree of susceptibility varies with the strains of rats (Bannon et al, 1945).

(b) <u>Diseases Caused by the Agent acting Postnatally</u>

The rat eye is variously described as being mature at 17 days (Bourne et al, 1938a, b) to 5 - 6 weeks (Lucas et al, 1955). The eye of the rat opens at 2 weeks of age, i.e. when it is immature and one would expect a gradual increase in function with the approach to maturity. Concurrent with this development there will be a gradual change in the pathology, from malformation due to agents acting before maturity, to inflammation and degeneration as a result of agents acting on the adult mature eye.

i) <u>Hereditary Conditions which are Expressed Postnatally</u>

For the purposes of classification these have been described under antenatal conditions ((a) (i)). It is difficult to separate this group as many of the hereditary malformations are not recognised until the rat eye opens at 2 weeks. However, reports of the effect of a hereditary condition on postnatal growth in the mouse have been published.

Quantitative studies on the visual cortex of mice with inherited retinal dystrophy revealed a retarded growth of cell nuclei in the striate area laminae 2, 3 and 4, but not in L5 and 6, over the period 20 - 60 days (Gyllensten and Lindberg, 1964).

Another non-quantitative morphological investigation into the visual cortex neurone density of mice with retinal dystrophy, examined at 1, 3, 5, 8 and 14 weeks indicated that there was no difference compared with controls (Mitra, 1957).

ii) <u>Non-Hereditary Postnatal Conditions</u>

These conditions are divided into the following headings: -

(α) Visual deprivation
(β) Enriched environment
(γ) Intense light
(δ) Chemical
(ε) Nutrition
(ζ) Viruses and Bacteria
(η) Carcinogens
(θ) Ageing changes

(α) <u>Visual deprivation</u> - There has been much research carried out on the effects of visual deprivation, and it is proposed to further divide this survey into the following headings:

A. Dark rearing - complete
   Partial

B. Monochromatic light rearing

C. En~~n~~ucleation

D. Eyelid suture - bilateral
   unilateral

The effects produced depend on the technique used, whether it is monocular or binocular, and the age at which it is performed. Species difference also plays a part - the higher the subjects position in the phylogenetic scale, the greater its susceptibility to the experimental treatment (Fifkova, 1967). The results obtained by visual deprivation vary with the parts of the visual system examined, the type of evaluation used and the units measured in quantitative work. By general observation no difference is visible in the visual cortex of a blind animal, only quantitative evaluation reveals a difference (Fifkova, 1967).

A. Dark rearing of the chimpanzee produces atrophy of the ganglion cells of the retina, reversible for the first few months, but thereafter irreversible. Although cats and rats fail to show this ganglion cell disappearance, there is a difference in the ribonucleic acid content of their ganglion cells, when dark reared and controls are compared (Riesen, 1960).

Another publication (Rasch et al, 1961) confirmed the degeneration of the ganglion cell layer in the dark reared chimpanzee but found a decrease in RNA and protein levels in retinae of cats and hooded rats which were light deprived from birth.

The amount of light per day is of importance - a few minutes of light per day did not prevent atrophy of the ganglion cell layer developing in a chimpanzee, whereas 1½ hours of diffuse light per day for 7 months was enough to stop the effect (Chow et al, 1957). Species, too, may be of importance as dark rearing a rhesus monkey (Macaca mulatta) which received only a few minutes of light per day from birth to 8 months did not produce any detectable change in the retina (Chow, 1955).

Mice, reared in darkness from birth to 5-30 days show a retardation in growth of the retinal ganglion cells and a delay in myelinisation of the optic nerve (Gyllensten and Malmfors, 1963).

Dark rearing mice from birth to 2 months of age produces a highly significant decrease in relative volume of internuclear material (an indication of cytoplasm volume) in the visual cortex, lateral geniculate body and superior colliculus. A decrease in visual cortex thickness in addition to a reduction in the diameter of the cell nucleus was recorded. Prolonging the time spent in darkness to 7 months tended to reduce the difference between controls and dark reared (Gyllensten et al, 1965).

Other measurements used in the cortex of light deprived mice have concerned the number of apical dendrite spines. The number of spines on L5 pyramidal apical dendrites in L4 was counted, and a significant reduction in the number of these spines was found in mice kept in total darkness from birth to 22-25 days (Valverde, 1968).

By keeping rats in darkness from birth to 3 weeks Cragg (1967) showed a decrease in the synaptic density in the lower half of the visual cortex when compared with controls. These electron microscope investigations also revealed a difference in size of the average synaptic diameter, with the controls having the larger synaptic diameter in the upper half of the cortex, and the dark reared a larger lower half mean synaptic diameter.

Dark rearing rabbits for 6 months produced a clear behavioural deficit but no effect on retinal thickness, optic tract or optic centres apart from a slight increase in density of cells in the superior colliculus, and of pyramidal cells in the visual cortex (Goodman, 1932) although 10 weeks of darkness produced changes in the concentration of ribonucleic acid and nucleoprotein in the ganglion cells (Brattgård, 1952).

Cats have also been used for sensory deprivation studies, a delay in maturation of the electroretinogram was observed in kittens reared in the dark (Zetterström, 1956). Kittens kept in darkness from 7 days to 14 weeks, and then masking one eye so that it received only diffuse illumination for 3 weeks, had thinner inner plexiform layers, and fewer Müller fibres in their eyes (Weiskrantz, 1958).

Metabolic changes in brain due to sensory deprivation have also been recorded. Mice reared in darkness and silence for 31 days were found to have decreased phospholipid turnover (Wase and Christensen, 1960). By keeping rabbits in darkness a reduction in protein mass in the nerve cells of the visual cortex was observed (Gomirato and Baggio, 1962).

B. Monochromatic light has also been used in visual deprivation studies. Monochromatic red light from birth caused selective laminar atrophy in two out of three monkeys (Clark, 1943).

C. Enucleation. Enucleation of the eyes of the rat at birth or at 4 months of age, was followed by a reduction in the size of the optic nerve and tract, volume reduction of both the dorsal and ventral portions of the lateral geniculate body, shortening of the anteroposterior length of the superior colliculus and slight reduction in lamina 2, 3 and 4 of the visual cortex, when the animals were killed at 7 months. Those animals allowed to survive until they were 17 months old exhibited slight withering of the cells of the visual cortex as an additional feature (Tsang,1937).

Enucleation of the rat eye at 14-15 days caused degeneration of the optic nerve and tract, with volume reduction, increased cell density and gliosis of the lateral geniculate body (Fifkova and Hassler, 1969).

Changes in weight and cholinesterase activity of the visual cortex of the rat after eye removal has also been demonstrated (Krech et al, 1963).

Mouse Enucleation in the mouse at birth, studied by the Golgi method produced degeneration of the optic nerve and tract. The enucleation also produces, through a series of transneuronal changes a decrease in the number of apical dendritic spines located in L4 and L3 (Valverde, 1968).

Enucleation of the eye of the mouse at 4-6 days old produced degeneration of the optic nerve and tract in 3-11 weeks with volume reduction of the stratum griseum, but no marked changes were detected in the geniculate body or visual cortex (Terry et al, 1962).

A reduction in cholinesterase content was found in the superior colliculus of the mouse after eye removal (Hess, 1960).

Rabbit and Cat Unilateral enucleation of the eye in the rabbit and cat produced a slow mild to severe atrophy of the lateral geniculate body (Cook et al, 1951). This work was repeated in the rabbit, again with atrophy in the contralateral geniculate body (Lindner and Umrath, 1955). The visual cortex was not examined in these experiments; one report of enucleation at 3 days of the cat failed to produce any changes in the visual cortex when examined at three months (Mitra, 1958).

D. Eyelid suturing as a means of visual deprivation has been used in rats, mice and cats. Suturing 14-15 day old rats for 8-9 weeks produced volume reduction in dorsal lateral geniculate body and the visual cortex, particularly L2-4, with an increase in cell density in L4 (Fifkova, 1967; Fifkova and Hassler, 1969).

Mouse eyelid, sutured from 4-6 days old for 3-11 weeks, however, failed to produce any changes in the visual system (Terry et al, 1962). Suturing the eyelids of kittens at 10 days of age and keeping for 3 months produced behavioural blindness and atrophy of the lamina of the contralateral geniculate body (Wiesel and Hubel, 1963).

In summary it can be seen that the wide variety of methods utilised for visual deprivation can often produce conflicting results. The different methods of evaluation of results, the different visual areas examined, and the many species used in this work also tend to confuse. However, the more refined techniques which are capable of revealing subtle changes have produced evidence that in the laboratory animals, visual deprivation from birth affects the entire visual system.

(β) Enriched Environment - The effects of enriched environment on the rat brain have also been investigated. By means of maze training and changing the maze barriers, rats received an enriched environment, which was found to produce a depth increase in the visual cortex, particularly of layers 2 and 3, and an increase in the intercellular and intervascular substance (Diamond et al, 1964). In addition further work indicated a 14% increase in glia (Diamond et al, 1966).

(γ) Intense Light - Applied for less than 1 hour or more moderate light for longer periods can damage the retina of the rat irreversibly, with degeneration of the pigment epithelium and rod cells associated with reduction in the amplitudes of the electroretinogram. This effect is very dependent on eye (body) temperature, hyperthermia accelerating and intensifying the damaging action (Noell et al, 1966). It is postulated that the visual cell, a highly differentiated, delicately balanced cell, is damaged as a result of a reaction to the sudden and prolonged step of cell activity from a level of functioning in dark or weak light to one of strong light. Vitamin A deficiency protects against this damage in the rat, and it is further postulated that retinol (Vitamin A) produced from excessive rhodopsin bleaching, acts as a toxic agent in the pigment epithelium (Noell and Albrecht, 1971), a mechanism very similar to that occurring in retinal dystrophy (Reading,1970).

(θ) Chemical Agents - Toxic changes to the eye may be caused by a wide variety of compounds (Grant, 1962). In humans the possibility of ocular side-effects of modern drugs has caused concern (Green and Spencer, 1969). Ocular toxicology comprises three main areas, the first covers the local effects of topically applied substances and the second concerns the systemic effects of local administration to the eye, a dramatic example of which is the almost immediate lethal action of certain war gases when applied to the eyes of experimental animals. The third area of study involves ocular side effects from drugs given by other routes (Marzulli, 1968).

In ocular toxicology the problem of species variation is of great importance. There are important structural and biochemical differences between the eyes of man and animals, and great care is required in extrapolation of results (Marzulli, 1968), and in fact even within the same species wide differences can occur. The rat is not an ideal animal for ocular toxicity (it is a nocturnal animal and not comparable with the human), but it is nevertheless the most commonly used laboratory animal (Heywood, 1973), and was used for the reasons stated earlier in Choice of Species. The drugs used in this work are not very effective on the rat retina (Noell, 1951; Goodwin et al, 1957); this was realised as it was hoped the system devised would be capable of recognising minimal changes.

In view of the large amount of literature on chemicals which affect the eye, it was decided to restrict the literature review to those drugs used in this work, i.e. Sodium iodoacetate and two diaminodiphenoxyalkanes, and the effect of an excess of environmental oxygen.

Iodoacetate intravenously was found to affect the rods, cones and pigment cells of the retinae of rabbits, cats and monkeys (Noell, 1952). Like oxygen poisoning and X irradiation, iodoacetate was highly selective for visual cells, principally the rods; causing pyknosis and chromatolysis, leading to disappearance of the visual cells with preservation of the other retinal layers (Noell, 1955; Noell, 1958). It is believed to act by inactivating triosephosphate dehydrogenase and so blocking glycolysis.

Secondary to iodoacetate induced retinal toxicity is a severe and widespread capillary degeneration throughout the retina of the cat. This secondary capillary degeneration has also been found after damage to the eye of the rat by light (Dantzker and Gerstein, 1969).

The diaminophenoxyalkanes are a series of compounds, which were first noted for their schistosomicidal activity (Collins, 1954). Clinical trials uncovered the retinal complications, and further examination revealed that the primary amino derivatives were the most toxic and tertiary compounds the least. Large doses caused intravascular haemolysis in mice and rabbits and loss of hair in mice in addition to retinal lesions (Goodwin et al, 1957). Ophthalmoscopically the picture produced was similar to iodoacetate - a loss of translucency of the retina appearing like oedema, narrowing of the arteries and pigmentary proliferation starting at 5 days and progressing for 2-3 weeks (Sorsby and Nakajima, 1958). In the cat, histologically swollen pigment epithelium with disruption and loss of the visual cells (Goodwin et al, 1957).

Further work on one of the series, 1:5-Di(p-aminophenoxy) pentane hydrochloride (May and Baker, 968A) showed a marked species variation in ocular toxicity to an oral dose with the monkey, dog and cat susceptible and not the mouse, rat, rabbit or guinea pig.

The pathogenesis of 968A toxicity was described in the cat affecting first the pigment epithelium, distorting the retina into arcades, then affecting the visual cells causing pyknosis of the outer nuclear layer and eventual disappearance. No gliosis or optic nerve changes occurred (Ashton, 1957).

The basis of action of this compound is thought to be a denaturing effect, causing a rise in total -SH groups of the retina (Reading and Sorsby, 1966).

A further interesting toxic effect on the eye is that of an excess of environmental oxygen. Newborn mice, exposed to 100% oxygen developed intraocular haemorrhages, retinal detachment with folding and the formation of blood vessels in the vitreous. These persistent proliferating retrolental hyaloid vessels retarded formation of the retinal blood vessels (Gyllensten et al, 1954). In addition there was a decrease in the striate area in the relative amounts of cortical vessels in all layers. After 10 days of oxygen exposure there was an increase in the average nuclear diameter and in the relative amounts of internuclear material in the striate area, and after 20 days a reversal of these findings, namely a reduction in nuclear diameter and internuclear material compared with controls (Gyllensten, 1959a, 1959b).

(ε) <u>Nutrition</u> - Vitamin A deficiency in the adult rat produces a range of changes depending on the duration and severity of the deficiency. Structural changes range from a mere alteration in staining properties of the outer segments of the rods to degeneration of several layers, involving in order the outer nuclear, pigment epithelium, outer molecular and inner nuclear layers (Johnston, 1943). The fine structure of this degeneration has been studied (Dowling and Gibbons, 1961).

(ζ) Viruses and Bacteria - Only a few viruses have been associated with rat eye abnormalities. Cataracts have been produced in mice by suckling mouse cataract agent (Clark, 1964) and in rats by St. Louis encephalitis particles (Hanna et al, 1968).

Dacroadenitis (Hunt, 1963), lacrimal gland and salivary gland inflammation, sialodacryoadenitis (Jonas et al, 1969) can involve the eye and cause conjunctivitis (Wagner et al, 1969). Purulent keratoconjunctivitis in rats due to Micrococcus pyogenes var. aureus (Foster, 1958) and panophthalmitis caused by Pasteurella pneumotropica in the mouse (Weisbroth et al, 1969) and Staphylococcus aureus in the rat (Jones, 1959) have also been described.

(η) Carcinogens - a Dermoid of a rat cornea recorded as a choristomatous tumour has been reported (Nichols and Yanoff, 1969). This is not a true tumour, choristomatous means histologically normal but in an abnormal site. In a report Saunders (1967) states that he has yet to see a convincing report of an ocular neoplasm in the rat.

(θ) Ageing Changes - Ageing changes in the lens revealing alterations of the anterior suture, opacities of the nucleus and posterior cataracts have been reported (Balazs et al, 1970, 1971; Heywood, 1973). An excellent paper on clinical observations on the rat eye reports a low incidence of spontaneous retinal lesions and a low natural occurrence of cataract (Heywood, 1973).

A retinal atrophy of unknown origin has also been described in Wistar Rat (Mawdesley-Thomas, 1968). There was gross distortion of the retinal layers, with loss of the outer nuclear layer and rods and cones layer. These changes were confined exclusively to males and were noted in 12/300 rats at the termination of the experiment, when the animals were between 18 and 24 months of age.

SECTION 4. SUMMARY

As can be seen from this literature survey there is a large range of agents and conditions which affect the visual system of the rat. The resulting pathology depends on many factors, one of the most important of which is the age or stage of development of the visual systems, as can be seen in the Malformation Timetables, Figs. 35-37, V.2.

The time of action of some agents can be determined exactly e.g. a single X-ray dose, whereas others such as trypan blue, hypervitaminosis A may have a precise time of administration but a much less precise duration of action. Gene action in hereditary malformations is presumed to act at the same time as non hereditary factors in producing comparable malformations.

In the context of this thesis the blind Campbell is the result of genetic action, and the resulting sequential changes which although not detectable until after birth can be regarded as a malformation and not an abiotrophy.

Since the defect in the Campbell is primarily in the eye one would expect changes in the visual cortex comparable with those produced by visual deprivation. Table A, page 51, summarises the literature in this respect and shows the lack of knowledge about the visual cortex, particularly quantitative results of the effect of damage if any resulting from retinal dystrophy.

An important time in the development of the formed visual cortex in the rat starts when the eyes open at 2 weeks and continues from then to adulthood with maturation of the visual system. Although the eye may be mature at 5-6 weeks (Lucas et al, 1955) the visual cortex may not mature until later so work on this aspect should cover a large enough period of the life of the rat to include maturation of the entire visual system.

The value of using the Campbell mutant is that a series of equally diseased models were available for comparing the visual cortex quantitatively with the normal P.V.G. If the system devised could show the minimal difference between the blind and normal then it might be of value in detecting minor damage due to drugs - particularly those drugs which have a minimal accumulative action. To detect these drugs long term tests are required and age changes, nutritional problems, spontaneous tumours, infections etc. can mask small changes, and interfere with interpretation of the results.

Table A

	Visual Deprivation	Retinal dystrophy
Eyes	Dark Rearing - Rat. No ganglion cell atrophy (Riesen, 1960) D.R. - Chimp. Atrophy ganglion cell (Riesen, 1960) D.R. - Rat. $\downarrow$ RNA) $\downarrow$ protein) retina (Rasch, 1961) D.R. - Mouse. Retardation of growth of ganglion cells (Gyllensten, 1963)	
Optic Nerve	D.R. - Mice. Delay in myelinisation (Gyllensten, 1963) Ennucleation - Rat. $\downarrow$ size (Tsang, 1937) En. - Rat. Deg. (Fifkova, 1969)	
Lateral Geniculate Body	D.R. - Mice $\downarrow$ internuclear material (Gyllensten, 1965) En. - Rat. $\downarrow$ volume. Dorsal & ventral (Tsang, 1937) En. - Rat. $\downarrow$ volume. $\uparrow$ cell density gliosis (Fifkova, 1969) En. - Mouse. No change (Terry, 1962) En. - Rabbit:Cat. Atrophy. (Cook, 1951) Lid suturing. - Mouse. $\downarrow$ vol. (Fifkova, 1967)	
Visual Cortex	D.R. - Mice $\downarrow$ internuclear material (Gyllensten, 1965) D.R. - Mice $\downarrow$ no spines on L_5 Δ apical dendrites (Valverde, 1968) D.R. - Mice $\downarrow$ phospholipid turnover (Wase, 1960) En. - Rat. $\downarrow$ L2,3,4. (Tsang, 1937) En. - Rat. $\downarrow$ cholinesterase activity (Krech, 1963) En. - Mouse. No change (Terry, 1962) L/sut. Mouse. $\downarrow$ vol. L2-4. $\uparrow$ cell density L4 (Fifkova, 1967, 1969)	Retarded growth L2, 3, 4 cell nuclei (mouse) (Gyllensten, 1964) No change in cell density (Mitra, 1957)

CHAPTER II

LITERATURE REVIEW ON C.N.S. QUANTITATION

		Page
SECTION 1.	STANDARDISATION OF HISTOLOGICAL PREPARATION	
	(a) The Fixative	53
	(b) The Technique of Fixation - Perfusion or Immersion	55
	(c) The Nature of the Tissue	56
	(d) The Technique of Processing	56
	(e) The Method of Cutting - Standardisation of Section Thickness	57
SECTION 2.	PROBLEMS INVOLVED IN PERFORMING THE COUNTS	
	(a) The Choice of Units to be Counted.....	62
	(b) The Methods of Counting	63
	(c) Delineation of Area to be Counted	64
	(d) Estimation of Cell Population in Area that is Counted	65
SECTION 3.	CORRECTION FACTORS FOR INHERENT ERRORS IN COUNTING	
	(a) Thickness Error	66
	(b) Counting Error	66
	(c) Split Cell Error	66
SECTION 4.	SUMMARY: PROTOCOLS FOR QUANTITATION	72
SECTION 5.	RESULTS FROM THE LITERATURE	73

CHAPTER II.

SECTION 1. STANDARDISATION OF HISTOLOGICAL PREPARATION

One of the first requirements for a quantitative histological assessment is the production of standard comparable stained sections. The volume of a fresh tissue specimen changes drastically during the histological procedures leading to finished sections (Stowell, 1941). To ensure parity of individual sections the factors affecting the dimensions and structure of the tissue must be recognised: these are

(a) The fixative - choice
- concentration, temperature and osmotic pressure of fixative

(b) The technique of fixation

(c) The nature of the tissue

(d) The processing techniques employed

(e) The method of section cutting

(f) The method of staining and mounting

(a) The Fixative

i) Choice of Fixative - The ideal fixative should preserve a tissue against microbial activity, osmotic damage and autolysis, and after removal of the fixative the specimen should remain an accurate representation of the living tissue; additionally the cells should retain their original size and no material should escape from the cells (Jones, 1972). He continues, however "It seems likely we must search for the most ideal of the non-ideal fixatives amongst the aldehyde fixatives."

Schultz and Case (1970) comparing fixatives found that glutaraldehyde fixes as far as it penetrates, unlike formaldehyde and acrolein. Formaldehyde as it penetrates faster than it fixes could influence morphological structure and modify it before fixation occurs. Nearly all fixing agents cause considerable changes in the weight of the brain, which depends on the properties of the fixing agent and the duration of its action on the brain; for example, 10% formalin causes a 25% increase in weight in the first 24 hours after immersion, whereas Bouins fluid does not alter the weight of the brain in this period (Blinkov and Glezer, 1968). Another factor is that the choice of fixative may be dictated by the subsequent techniques as for many histochemical methods. Many fat stains for example require non alcoholic fixatives.

The chosen fixative should be suitable for as many subsequent techniques as possible. Absolute standardisation of preparation and fresh solutions are essential for quantitative work.

ii) <u>Concentration, temperature and osmotic pressure of the fixative</u>. Hypertonic solutions such as 37% formaldehyde cause marked brain volume shrinkage when perfused (Schultz and Case, 1970) whereas weaker solutions (3-4% formaldehyde) cause brain swelling when perfused (Frontera, 1958). From a study of the literature and by experimentally measuring the effect of different concentrations and osmotic pressures of fixatives. Frontera (1958) concluded that the above factors were responsible.

(b) <u>The Technique of Fixation</u>

<u>Perfusion or Immersion</u> - Whenever possible fixation by perfusion is preferable to fixation by immersion (Cammermeyer, 1967) to avoid the crush cell artefact or dark neurone, for more reproducible standard results and to minimise the number of artefacts. Fixation must begin as soon as possible after death as Trump and Ericsson (1965) found that postmortem change (autolysis) is preceded by an increase in fragility which may predispose to processing damage. Schultz and Case (1970) noted that any delay in the onset of perfusion resulted in a slow rate of flow of perfusion and an increase in fixation artefacts. The perfusion will require an adequate volume to completely fix the tissue and sufficient pressure to thoroughly flush out all the blood.

The problems of immersion fixation are due to distortion during fixation and the uneven fixation due to block size where a crust of fixed tissue can form with a centre of unfixed material (Zeligman, 1946). The time spent in fixative is also of importance in that larger blocks of tissue will require a longer immersion than smaller blocks.

Thus the preferred technique is to perfuse the anaesthetised animal, and if this is not possible to immersion fix as soon as possible after death. The fixative and technique chosen are described in Chapter III; in short, a three solution perfusion was used, the first a tyrode solution to flush out the blood, the second a concentrated (19%) glutaraldehyde to fix the tissue immediately but only to act for seconds, and finally the main perfusate solution to stabilise the tissue. This procedure is believed to cause little or no shrinkage (Schultz and Case, 1970).

(c) <u>The Nature of the Tissue</u>

The chemical composition of the tissue will affect the final result - the high lipid content of the central nervous system will shrink more during alcohol dehydration than other tissues.

The water content of the brain varies with age, 90% newborn, 70-80% adult (Heinze, 1954) and this will again produce a different final result.

Individual differences and disease of blood vessels can also affect the fixation and hence the dimensions and structure of the tissue.

(d) <u>The Technique of Processing</u>

In processing material to paraffin blocks, solvents are used to remove water and lipid from the tissue. This extraction will depend on the state of solubility, temperature and the duration of action of the solvent. Moreover the repeated use of solvents results in them becoming saturated with water and lipid, hence the degree of extraction will vary.

The time spent in xylol affects the thickness of the section as shown by Treff (1963), who found that sections were reduced in thickness by 5% after an hour, and by 22% after 48 hours in xylol.

Thus it can be seen that a common source of error in quantitation of the central nervous system will be caused by the variations in preparation of sections. Many authors (Haug, 1972) although admitting that the shrinkage caused by fixation and embedding must be taken into account, tend to use a standard shrinkage (about 50%) and state that there is no necessity to go into details.

However, one of the few authors to use a correction factor for shrinkage due to section preparation (Pakkenberg, 1966) found a mean linear shrinkage of 36%, which was equivalent to a volume shrinkage of 74% and shows just how much variation is possible when compared with the standard 50% of Haug (1972).

Staining and mounting will produce the same problems, i.e. solvent solubility, temperature and duration of action. Standard preparation of stains is required. As different stains vary in staining sensitivity and intensity, one stain alone should be used preferably one with a high degree of repeatability. The mountant used may contract with time again affecting the section. Some of these factors such as block size will also apply to araldite blocks used in electron microscopy.

(e) <u>The Method of Cutting</u>

<u>Standardisation of section thickness</u> - Many thicknesses have been used in quantitation of nervous tissue, ranging from 5μ of Gyllensten (1960) to 50μ of Chow (1951). For neuroanatomical quantitation, ideally the thicker the section the better - the

smaller the split cell error (which thus reduces the risk of counting the same structure twice in serial sections) and the smaller the number of sections required. However, non recognition of structures and inaccurate measurements of these structures are possible due to the large depth of focus.

For measurements as accurate as possible of all the cell types present in the visual cortex a thickness of 6μ (microtome setting 5) was used in this work. (This setting was equivalent to a range of unprocessed block thickness of 9-15μ as explained under Reduction Factor (Chapter III, Sect. 4)). A correction factor could be used to allow for this thickness where the structural unit is of the same magnitude or even larger than the section thickness (Weibel, 1965).

In addition the microscope objective lens had a N.A. (numerical aperture) of 1.3 and this results in a somewhat limited depth of focus as calculated by Berek's formula (Haug, 1956).

$$T = n_o \left(\frac{4\lambda k}{A^2} + \frac{S}{AV} w \right)$$

where T = depth of focus

n_o	= Index refraction object examined	= 1.45μ
λ	= Wavelength light	= 0.55μ
k	= Constant	= 1/8
A	= Aperture of objective	= 1.3
S	= Conventional range vision	= 250,000μ
V	= Total magnification microscope	= 2478
w	= Constant	= 0.00136

The microscopic objective focal length was found to be only 0.39μ, and as the subjective depth of focus (i.e. the depth of focus extended by the after accommodation of the eye) is 0.5μ, it is obvious that thicker sections will involve a great deal more fine focusing than thinner sections.

It is essential to know the section thickness and to check this thickness for error. Several workers have demonstrated a variance between the measured section thickness and the microtome setting (Graf, 1948; Marengo, 1944). To verify the thickness of a section there are five methods:

(a) surface focusing - using the fine focus on the microscope on the upper and lower surface of the section.

(b) direct measurement of cross section - a section is re-embedded and cut at right angles to the usual direction and measured with a calibrated eyepiece graticule.

(c) interferometric - light is reflected from the two surfaces of the section onto a spectrometer, and the difference between the two surfaces calculated from the bands produced in the spectrum.

(d) light profile - light at 45° is projected on the section, and reflected from both surfaces, the difference between the two reflected beams being equal to the section thickness.

(e) Stereoscopic - the section is tilted so that the section is in focus throughout the thickness of the section. This technique is used more in electron microscopy.

The last three methods require special equipment (Lange and Engstrom, 1954). The section thickness error is greater with thin (5 - 10μ) sections and constant checks were made during the course of this work to ensure parity, using surface focusing and direct measurement.

SECTION 2. PROBLEMS INVOLVED IN PERFORMING THE COUNTS

These factors will be dealt with in the following order:

(a) Choice of Units to be Counted

Cell Body (perikaryon); nucleus; nucleolus.

(b) The Methods of Counting

i) Photographic

ii) Projection

iii) Microphotometric

iv) Homogenate

v) Ocular

(c) Delineation of the Area to be Counted

(d) Estimation of Cell Population in Area that is Counted

Total Count v.s. Sample Count.

(a) <u>The Choice of Units to be Counted</u>

The neuronal cell body, its nucleus or its nucleolus have been used as the unit counted in neuroanatomical quantitation, based on the assumption that each neurone has only one nucleus and one nucleolus. Binucleated neurones are seen occasionally especially in some diseases, e.g. tuberous sclerosis and extremely rare neurones with more than one nucleolus have been seen (Konigsmark, 1970).

The cell body or perikaryon was used as the unit in counts of the lateral geniculate body in man (Sullivan et al, 1958) without any correction factor for split neurones. Escobar et al (1968) compared the cell body and nucleoli counts in twenty successive sections and concluded that the nucleolus cell count x 2 = cell body count, and used the cell body count. To estimate the split cell error Bok and Kip (1940) used sections of 15 and 30μ.

Neuronal nuclei have been used as the unit for counting (Guild et al, 1931; Chow, 1951; Irving and Harrison, 1967) although only Guild made a correction for split cell nuclei of about 10%.

With glial cells, information is somewhat sparse. Changes in the glia/neurone index postnatally (Brizee et al, 1964) using the Chalkey method (Chalkey, 1943, 1949) involved counting the numbers of both neurones and glia. By using photographs of 10μ sections and differential cell counts Diamond et al (1966) counted both neurones and glia in the rat cortex. These authors quantitated cell density, and did not measure cell size.

(b) The Methods of Counting

The methods outlined below have been used principally for the counting of neurones, and rarely for glia. The techniques include:

i) Photographic

ii) Projection

iii) Microphotometric

iv) Homogenate

v) Automatic

vi) Ocular

i) Photographic Photographs are taken of a section and cells counted on the photograph. This method has been criticised (Agduhr, 1941; Rowland and Mettler, 1949) because of the difficulties of focusing. The photograph is only dependent on the limited depth of focus of the microscope, and those cells outside this plane of focus will appear as blurred vague outlines. Photographs lack the microscopists subjective depth of focus.

ii) Projection The histological section is projected and the cells counted and measured. This method has been used by several authors (Dornfield et al, 1942; Sullivan et al, 1958; Escobar et al, 1968). In this system the microscopist adjusts the fine focus so that all the cells in the section are brought into sharp focus.

iii) Microphotometric (Ryzen, 1956). A moving light passing through a section and then directed onto a photo cell. The photocell readings were affected by the size and density of the cells in the section and a coefficient was calculated by dividing the photo cell readings (the optical density) by the cell numbers. Once the standard was obtained, cell numbers in other sections could be obtained, although the coefficient would depend on section thickness and stain intensity.

iv) Homogenate Method Suspensions of brain are diluted and counted in a haemocytometer chamber, where differential counts are made (Nurnberger and Gordon, 1957). This method permits reasonable estimates of total cell number per gram weight but the differential count is open to gross error (Konigsmark,1970).

v) Automatic Counting Devices The flying spot microscope (Causley and Young, 1955), and the Quantimet (Cole and Bond, 1972) have been developed to automate the slow laborious process of counting particles. Problems of size discrimination for irregular shapes, cell type recognition and cell overlapping have arisen, but when solved these machines will be of much value for quantitation.

vi) Ocular This has been the most frequently used method, the section being examined under a microscope, fitted with a measuring graticule, image splitting eyepiece or other measuring device.

(c) Delineation of Area to be Counted

In quantitation of the nervous system the problem of the extent of the area to be investigated arises, i.e. defining the borders. There are many areas, e.g. nuclei, cortical lamina where delimitation is difficult and standard procedures must be produced for this purpose. Furthermore for comparison of results in the literature it is important that the criteria used to define the area counted should be clearly stated.

(d) Estimation of Cell Population in Area to be Counted

There are four different methods for estimating cell population of a structure. These are :

i) Total Count

ii) Systematic Sample

iii) Random Sample

iv) Sample and Volume Method.

i) Total Count - All the cells are counted in serial sections through a structure. This method has been used for small discrete nuclei in the brain, e.g. the human motor trigeminal nucleus (Lassek, 1940; Tomasch and Malpass, 1958; Konigsmark et al, 1969).

ii) Systematic Sample - A systematic sample of sections throughout the structure is quantitated.

iii) Random Sample - A sample of sections is picked by means of random numbers and quantitated.

iv) Sample and Volume - This differs from (ii) and (iii) in that the volume of the structure is also obtained by quantitative means.

SECTION 3. CORRECTION FACTORS FOR INHERENT ERRORS IN COUNTING

When counting from sections, in addition to the section thickness error already described, there are two further sources of error - namely the Counting Error (reliability of count) and the Split Cell Error.

i) Section Thickness Error - The sections being quantitated may vary in thickness - see Chapter II, Sect.1 (e).

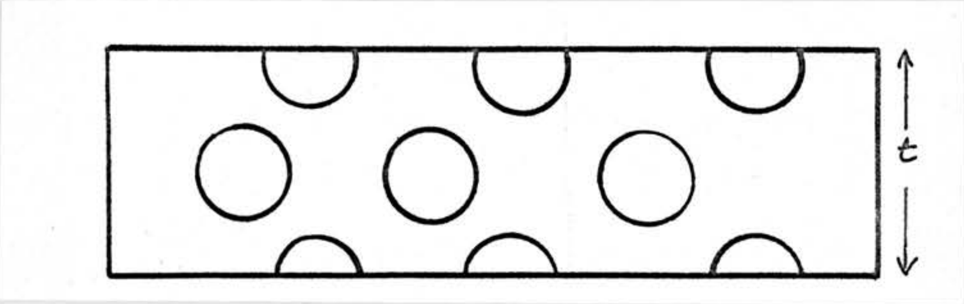
ii) Counting Error - Repetitive counts of the same population can produce degrees of discrepancy due to human error ranging from 2-10% (Gardner, 1940; Duncan and Keyser, 1936). A recount check should therefore be built into the system of quantitation used. This does not eliminate the error, but it reduces it, as the researcher is stimulated to minimise carelessness. In addition the figures obtained help to establish a guide as to what differences in cell number are likely to be significant.

iii) Split Cell Error - In a section of thickness "t" as well as complete cells there will also be portions or fragments of cells which have been cut during section preparation. These fragments when quantitated will be counted as complete cells although ideally they should be regarded as fractions.

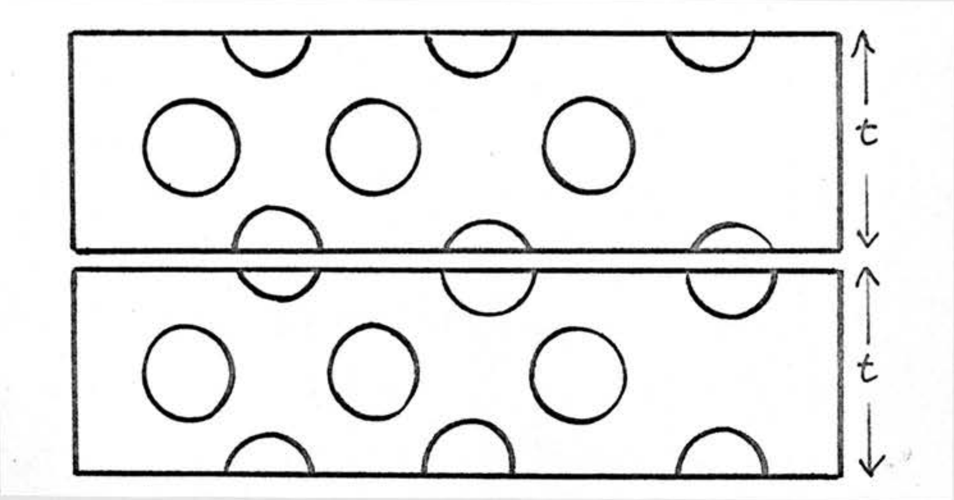
Correction factors have been developed to overcome this problem, used for any spherical unit, cell body, nucleus or nucleolus (Agduhr, 1941; Abercrombie, 1946; Floderus, 1944).

Development of Split Cell Correction Factor.

In quantitation of microtome sections of thickness "t", whether the unit counted is the cell body, nucleus or nucleolus, there will always be an overcount due to split cells near the surface of the section being counted as complete cells.



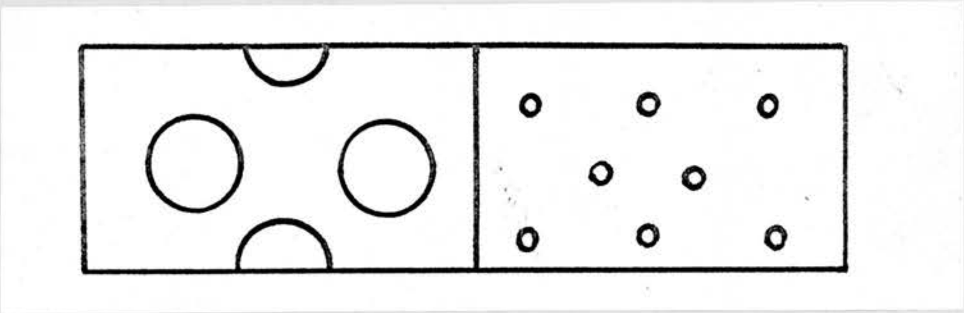
The microscopist examining the above section would estimate the total of cells as 9, instead of the correct result of 3 complete cells and 6 fractions. Furthermore, if serial sections were being counted as below:



Microscopists total = 18.

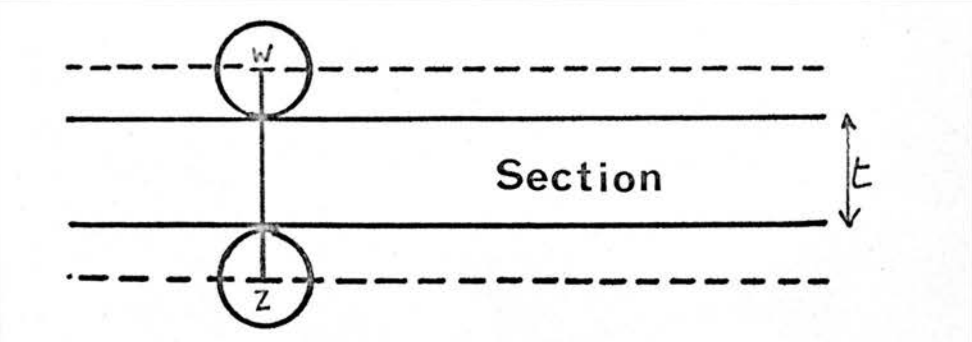
Correct result = 9 complete cells and 6 fractions or fragments.

To overcome this problem correction factors have been developed, based on the diameter of the unit counted and on the section thickness, and assuming a random distribution of spherical units.



This diagram shows that the larger the unit counted the greater the split cell error. With both large and small cells present an average value is taken to estimate the correction factor.

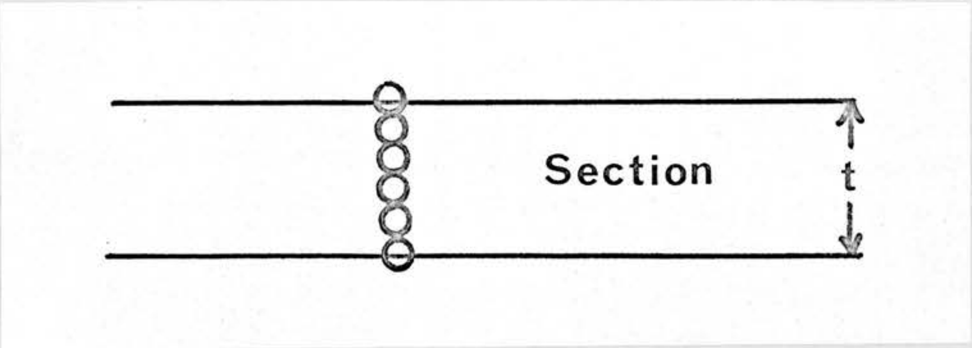
Abercrombie (1946) suggested that the true population or density of cells in a section could be calculated by proportion. Diagrammatically in a section of thickness "t", any cell, the centre of which lies between the lines "w" and "z" will be counted where w + z lie a distance of the radius of the cell from either surface of the section.



If we assume a random distribution of cells, then the proportion of cells which lie in section thickness "t" is given by distance $\frac{t}{wz}$

$$\therefore \frac{\text{True count} \quad c}{\text{Microscopists count } C} = \frac{\text{thickness}}{\text{thickness} + 2\ (\text{radius of cell})} = \frac{t}{t + 2r}$$

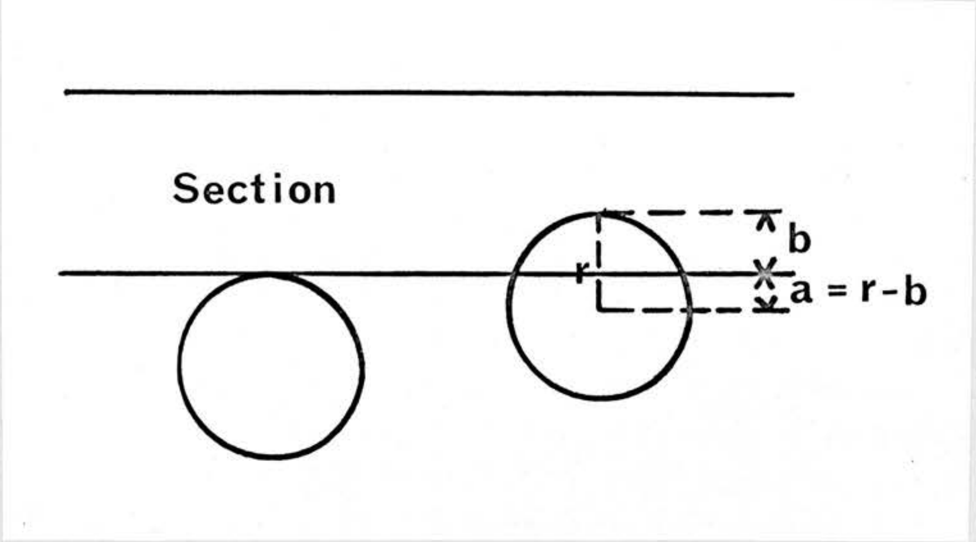
Example of Abercrombie :



$$\frac{\text{True count} \quad c}{\text{Microscopists count } C} = \text{Proportion of thicknesses} = \frac{t}{t + 2r}$$

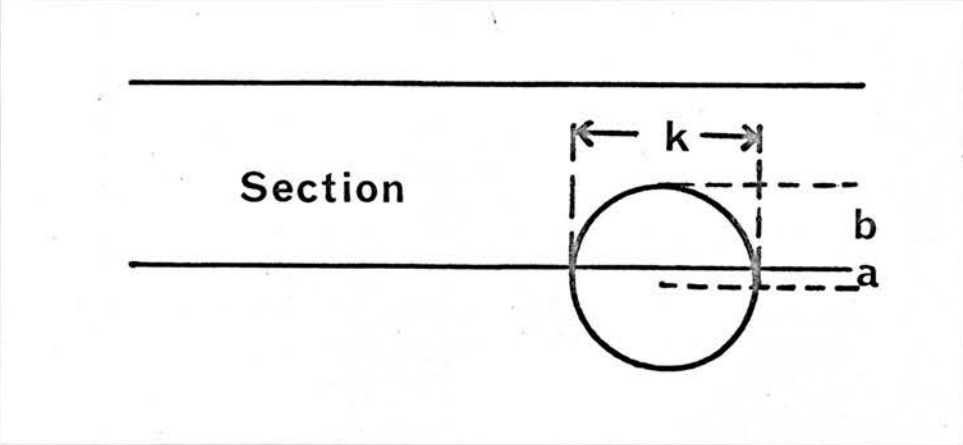
$$\frac{c}{C} = \frac{t}{t + 2r} \qquad \frac{c}{6} = \frac{10}{10 + 2} \qquad c = \frac{60}{12} = 5.$$

This is a highly diagrammatic example, but it serves to explain how the correction factor reduces the microscopists overcount. Floderus (1944) independently developed another correction factor. The microscopist does not see cells which are just touching the surface of the section but only those which extend a distance "b" into the section.

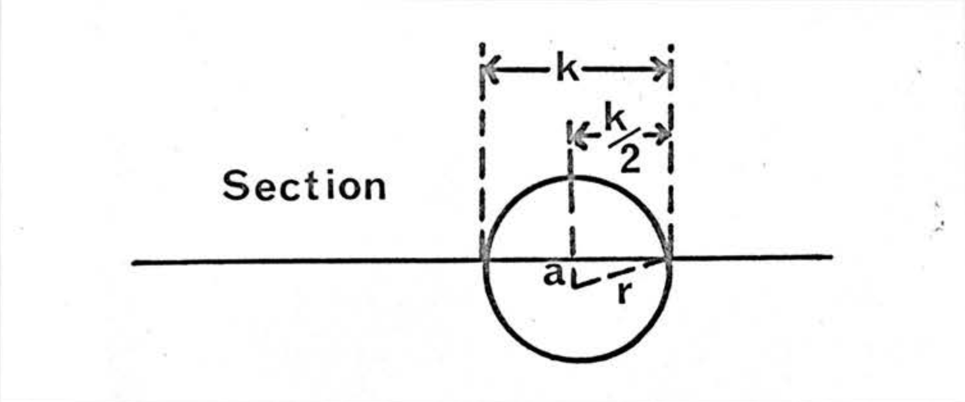


$$\therefore \frac{\text{(True)} \quad c}{\text{(Microscopists) } C} = \frac{t}{t + 2a} = \frac{t}{t + 2r - 2b}$$

Murphy (Konigsmark et al, 1970) however pointed out that the microscopist does not see the height "b" of the fragment, what in fact is observed is the linear dimension "k" of the fragment.



So what the correction factor needs is the distance "a" (r - b) expressed in terms of "k".



This distance "a" can be found by using Pythagorean theorem. From the diagram "a" is one side of a right angle triangle so that

$$r^2 = a^2 + \left(\frac{k}{2}\right)^2$$

$$a^2 = r^2 - \left(\frac{k}{2}\right)^2$$

$$a = \sqrt{r^2 - \left(\frac{k}{2}\right)^2}$$

and again this applies to both sides of the section:

$$\therefore\ 2a = 2\left(\sqrt{r^2 - \left(\frac{k}{2}\right)^2}\right)$$

Abercrombie $\frac{c}{C} = \frac{t}{t + 2r}$

Floderus $\frac{c}{C} = \frac{t}{t + 2r - 2b}$

Murphy $\frac{c}{C} = \frac{t}{t + 2\left(\sqrt{r^2 - \left(\frac{k}{2}\right)^2}\right)}$

None of these correction factors are ideal, as the basic assumptions of uniform distribution of spherical units in the cerebral cortex is not met. However, they do serve to produce a more accurate result for cell density than if they were ignored.

SECTION 4. SUMMARY

PROTOCOL FOR QUANTITATION

This chapter shows there are so many variables in quantitation that standard procedures must be developed and correction factors produced as shown below:

(a) Section preparation variables

(i) Fixative (ii) Processing (iii) Section thickness (iv) Staining	Standardise: Processing Correction Factor

(b) Quantitation variables

(i) Units counted (ii) Method of counting (iii) Sampling method of area to be counted	Standardise: Split Cell Correction Factor.

SECTION 5. RESULTS FROM THE LITERATURE

A summary of the quantitative results and techniques in the literature by Konigsmark (1970) was revealing, in that out of 35 reports only 2 related to the rat, and these 2 were concerned with the spinal ganglia. However, in addition there is a useful paper by Brizee et al (1964) on cell density in Area 2 (Kreig, 1946) of the rat cortex, found by three methods -

(α) Chalkey method - histological counting by means of a grid graticule

(β) Desoxy ribonucleic acid phosphorus method - the total D.N.A.P./g. tissue is divided by the amount of D.N.A.P. per nucleus

(∝) Cell maceration method - the cortex is macerated and the destruction rate of cells plotted, and extrapolated back to zero time.

ADULT RATS

Method	Neurone Density		Neuroglia Density	Total Density	
				Neurones + Glia	Total Cells
α	Fixed	90,000	50 - 85,000	140,000	230,000
	Fresh	-	-	-	106,000
β		-	-	-	112,000
∝		20,000	55,000	75,000	-

These results will be compared with those found in this work in the discussion (Chapter 8).

CHAPTER III

MATERIALS AND METHODS

Page

SECTION 1. AIMS OF METHOD 75

SECTION 2. CAGES AND HUSBANDRY OF EXPERIMENTAL ANIMALS 76

SECTION 3. PREPARATION OF STANDARD SECTIONS

(a) Fixation 77

(b) Blocking 80

(c) Photography 82

(d) Processing 83

SECTION 4. QUANTITATION

(a) Reduction Factor 87

(b) Visual Cortex Depth 91

(c) Counting and Measuring 92

SECTION 5. COMPUTER ANALYSIS AND TREATMENT OF DATA 97

SECTION 6. ELECTRON MICROSCOPY 103

SECTION 7. EXPERIMENTAL DRUGS USED TO TEST FOR RETINOTOXICITY 105

CHAPTER III

SECTION 1. AIMS OF METHOD

The aim of this work was to produce standard preparations of visual cortex of blind and sighted rats in such a way that any inevitable variations in processing etc. could be allowed for. It is important in quantitative work that once methods have been standardised, these methods must be strictly adhered to if comparable results are to be obtained.

It was decided to quantitate firstly the development of the normal and abnormal visual cortices and secondly, after development and maturation the adult blind and sighted cortices. Any difference revealed by the quantitative investigation would then be further explored using special stains.

The other purpose of the work was to attempt to reveal the cortical effects of challenging the rat with drugs which have a very low retinotoxicity in this species.

SECTION 2. CAGES AND HUSBANDRY OF EXPERIMENTAL ANIMALS

The colony of P.V.G. and Campbell rats was started using two sets of five breeding pairs which were kept under identical conditions. An old-fashioned animal house with natural daylight was used, until July 1972, when the rats were changed to a new constant environment building, having a temperature of 21°C, 50% humidity with 8 hours light per day and constant supplementary corridor lighting. Polypropylene cages (North Kent Plastic) types RB 3 and RC 1 were used, the former for breeding pairs and the latter for stock. Food (Oxoid Breeding Diet) and water were provided ad lib., with whitewood shavings and Spildri (Oakite Ltd) employed as bedding.

An inbred full-sib breeding system was adopted, usually brother sister although occasionally an experienced male was used with virgin females in cases where mating failed to occur, returning to brother sister after the first litter. Breeding was found to be slower with the Campbell strain, and the average litter size of this strain was also lower, seven as against ten for the P.V.G.

Three rabbits were also used in the course of this work and these animals were kept in the old type natural daylight animal house in aluminium cages with grid floors (Forth-Tech Services Ltd). Food (Oxoid S.G.1 diet) and water were provided ad lib.

Ophthalmoscopy was carried out at regular intervals on these experimental animals and before every perfusion. The pupil was dilated with a mydriatic (0.5% Mydriacyl-Tropicamide-Alcon) which took effect within a few minutes and the eyes were examined in a darkened room. A "Keeler Practitioner" hand held ophthalmoscope was used, usually at a setting of + 11 D.

SECTION 3. PREPARATION OF STANDARD SECTIONS

(a) Fixation

(b) Blocking

(c) Photography of block

(d) Processing.

(a) Fixation - It was decided because of more reproducible results and to minimise artefacts to use a perfusion technique for fixation. A modified aldehyde perfusion technique (Schultz and Case, 1970) for electron microscopy was utilised to prepared fixed tissue for both light microscopy and electron microscopy.

Perfusion procedure - rats handled as gently as possible were lightly anaesthetised with Diethyl ether (British Drug Houses Laboratory reagents) and then weighed. While the rat was still under ether anaesthesia an injection of pentobarbitone sodium (Nembutal - Abbot Laboratories Ltd) was made intraperitoneally at a dose rate of 35.2 mg/kg (Nembutal Veterinary Solution for small animal anaesthesia), and a satisfactory level of anaesthesia was usually induced in five to ten minutes as shown by the absence of the tarsal reflex on pinching the hind feet.

The abdomen and thorax of the rat was then opened and the posterior aorta and posterior vena cava clamped off with curved artery forceps. A 16 gauge needle connected to polythene tubing of external diameter ½ cm. was inserted in the left ventricle and the right atrium was excised. The following solutions were allowed to flow into the circulatory system of the animal, at a standard pressure produced by having the drip bottle 5 ft above the animal. This distance of 5 ft was equivalent to a pressure of 75 mm Hg.

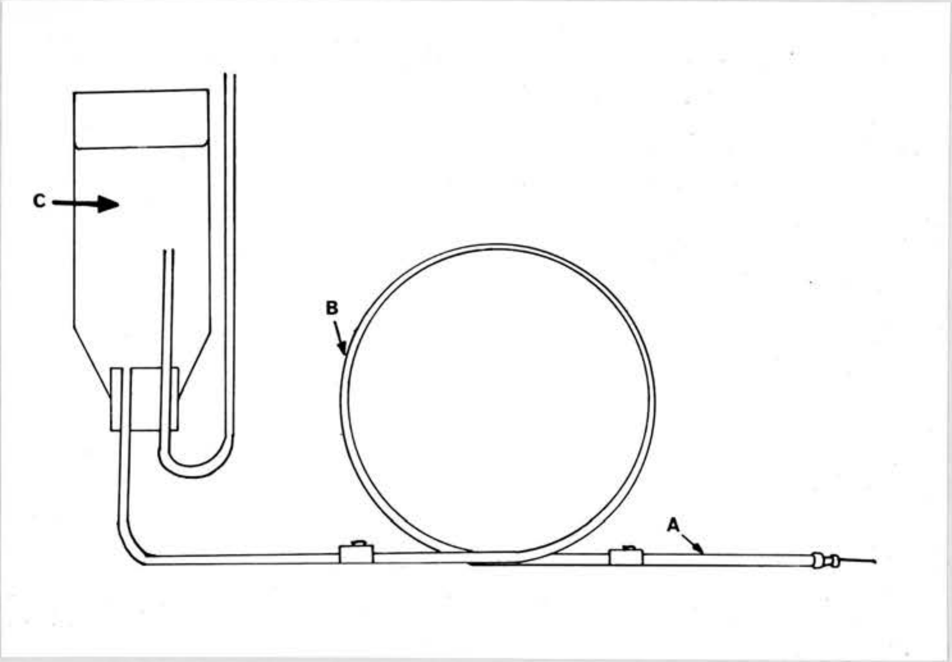


Diagram A.

PER RAT

Solution A : 10 mls - Tyrode solution with 1% gum acacia added.

Solution B : 15 mls - Phosphate buffered 19% glutaraldehyde.

Solution C : 250 mls - Phosphate buffered 2% glutaraldehyde.

Details of solutions:

Solution A: 97.5 mls Tyrode solution

2.5 mls 20% gum acacia.

<u>Solution B</u> : 18.76 mls stock buffer

5 mls 20% gum acacia

0.8 ml polyphosphate

75.64 mls 20% glutaraldehyde.

<u>Solution C</u> : 25 mls Stock buffer

5 mls 20% gum acacia

12 mls 20% glutaraldehyde

2 mls polyphosphate

56 mls distilled H_2O.

<u>Tyrode solution:</u> 0.5g Nacl

0.02g Kcl

0.02g $Cacl_2$

0.021g $Mgcl_2.6H_2O$

0.007g $NaH_2PO_4.2H_2O$

0.1g $NaHCO_3$

0.1g Dextrose

in 100 mls distilled water.

<u>Stock buffer:</u> 3.746g $Na\ H_2\ PO_4.2H_2O$

46.117g $Na\ H\ PO_4.12H_2O$

in 1000 mls distilled water.

<u>Polyphosphate solution:</u> 179.2g waterglass

1.2 mls 0.1 N NaoH

in 1000 mls distilled water.

(Schultz and Case, 1970).

These solutions were kept in a fridge until just before perfusion to maintain a constant temperature. The complete perfusion occupied

10 - 15 minutes after which the eyes and brain were carefully dissected out. The eyes were then immersed in Davidson's fluid (20 parts 40% formaldehyde, 10 parts glacial acetic acid, 30 parts 95% I.M.S., 30 parts distilled water) for 24 hours.

After the perfusion a macroscopic examination of the rat was carried out to ensure that the animal was healthy. Those animals which showed signs of middle ear disease were culled from the colony and if this condition was discovered on brain dissection the specimen was discarded.

With the drug experiments a full post mortem was carried out, with histological examination of lungs, heart, liver, kidney, sex organs and gut. These tissues were immersion fixed in 10% neutral buffered formalin (Culling, 1963) for 1 - 2 weeks.

(b) Blocking - Immediately after dissection the perfused brain was cut into blocks. To ensure repeatability a system was devised to make a standard cut in all the brains processed, despite great variation in size. A glass plate negative of the dorsal view of a rat brain was produced and a line drawn in representing the long axis (Diagram B). The area of the cortex comprising the visual cortex, the striate area was known from the map of Kreig (1946a) so a line was drawn on the glass plate negative, at right angles to the long axis, and dividing the striate area into an anterior 1/3rd and a posterior 2/3rds:

The resulting negative appeared, with the key cut X-Y outlined.

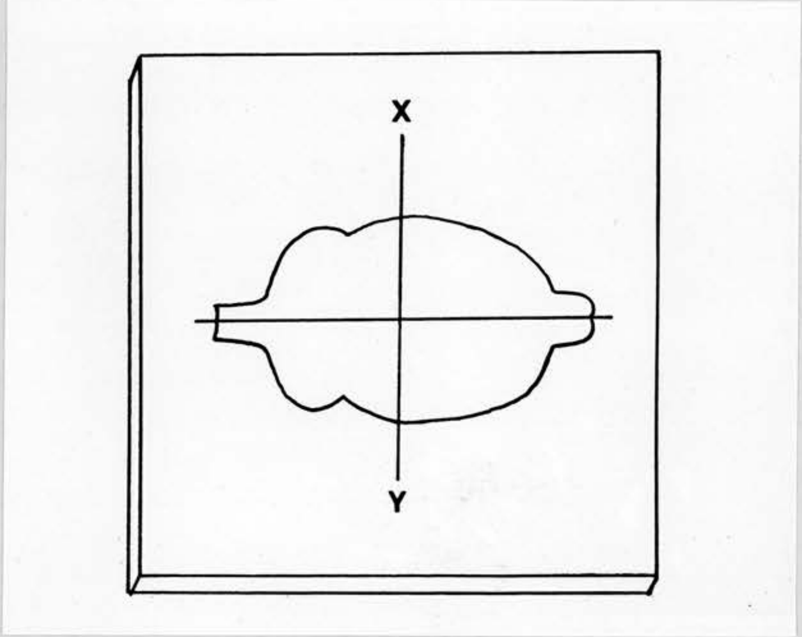
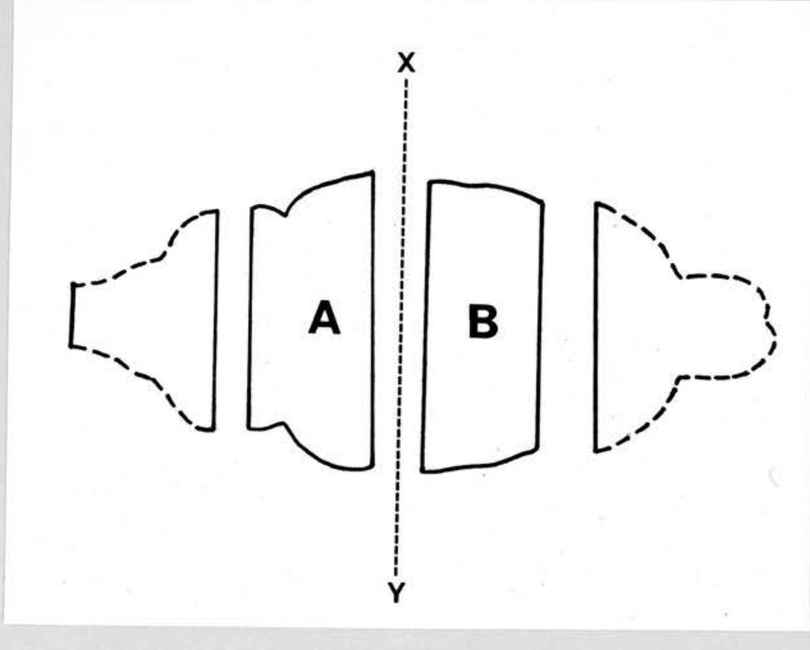


Diagram B.

The negative was placed in a "Kodak Precision Enlarger" and adjusted so that the image produced was equal in size to that of the brain to be sectioned. Hence this important cut was proportionally in the same region every time.

Further slices were made in the brain so that ultimately two blocks A and B were prepared (as in Diagram C).



The eyes, whether perfused or not, were immersed in Davidson's fluid for 24 hours to harden before cutting. Initial trials with liquid nitrogen proved unsatisfactory. The cut was made with a sharp razor slicing off a calotte and removing the large lens with forceps. The rat eye was then examined for any macroscopic findings after which the eye block was kept in 70% alcohol until processing.

(c) Photography of the Block - In order to estimate the variation due to the inevitable differences in processing a reduction factor (R.F.) was produced. The perfusion method of Schultz and Case (1970) is believed to cause little or no shrinkage so the perfused brain was taken as the standard. To make a permanent record of the dimensions of this standard photographs of the blocks described above were taken. These negatives could then be compared with the stained mounted section to find the degree of reduction.

The procedure immediately after blocking the perfused brain was to place each block on a glass plate, with a m.m. scale at the same level as the face of the block and photograph each face.

These photographs were taken using a Nikon F camera 55 mm f/ 3.5 with lens extension ring, at a stop of 11, time 1/15 sec. using Ilford PAN F film (ASA 50 DIN 18).

The distance was such to give a negative/subject mag. of 1:1. Developing was performed with ID11 in water 1:1 for 10 mins and acid fixer for 10 mins.

After photography the blocks were placed in baskets and immersed in 70% alcohol until processing. Initially an attempt was made to standardise the time in alcohol (1 week) but the variation in material prevented this, and any size difference due to varying the alcohol immersion time would be noticed when photographs of the stained section were taken and compared with the perfusion photograph (see Reduction Factor).

(d) Processing - A double embedding method was used for both eye and brain blocks as this allowed both sets of tissues to be processed together.

The schedule used was as follows :

1.	80% meths	2 hrs	)
2.	74 o.p. spirit	4 hrs	)
3.	74 o.p. spirit	2 hrs	)
4.	Absolute alcohol	2 hrs	)
5.	50:50 abs. alc.: amyl acetate	1 hr	)
6.	Amyl acetate	4 hrs	) Total time
7.	Amyl acetate	4 hrs	) = 47 hours.
8.	5:95 celloidin : methyl benzoate	9 hrs	)
9.	5:95 do. do. do.	9 hrs	)
10.	Benzene	1 hr	)
11.	Paraffin wax	4½ hrs	)
12.	Paraffin wax	4½ hrs	)

When processing had finished, the tissues were vacuum embedded in paraffin wax at 60°C for 20 minutes at a vacuum of 15"Hg. After vacuum embedding the tissues were embedded using "Tissue-Tek" moulds and embedding rings.

The wax used was Fibrowax - melting point 57-58°C (Raymond Lamb).

The sections were cut on a Leitz "Minot" rotary microtome at a setting of 5 on the "section thickness knurled knob setter". The importance of a constant thickness was realised so the technique of Marengo (1944) (where sections floated out on a water bath are re-embedded and cut at right angles for direct measurement) was performed continually.

Sections were floated out on egg albuminised slides and left for 24 hrs in an incubator at 37°C. Sections were taken of block A (Diagram C) at the level of the lateral geniculate body. A total of 9 sections were floated out on 3 slides giving 18 areas of visual cortex for each animal.

For staining Einarson's (1932) Gallocyanin stain was used as follows :-

1. Xylene .... 2 mins
2. Xylene .... 2 mins
3. Abs. alcohol .... 5 mins
4. 95% alcohol .... 5 mins
5. 50% alcohol .... 5 mins
6. Dist. H_2O .... 2 mins
7. Dist. H_2O .... 2 mins
8. Gallocyanin soln .... 48 hrs pH 5.
9. Dist. H_2O .... Rinse
10. 50% alcohol .... 2 mins
11. 70% alcohol .... 2 mins
12. Abs. alcohol .... 2 mins
13. Xylene .... 5 - 15 mins
14. Mount in DP.X.

Gallocyanin which stains nucleic acids was chosen because it is a progressive stain, and since differentiation is unnecessary it is a good method when sections are numerous. The dye solution will keep only for a month so fresh solutions were made every week, and the sections were allowed the maximum time in the stain (48 hrs) to ensure all the nucleic acid that would react was stained.

Other special stains were used in the course of this work.

<u>Paraffins</u>

Cresyl fast Violet (Drury and Wallington, 1967)

Goldner's Trichrome (Disbrey and Rack, 1970)

Haematoxylin and Eosin (Drury and Wallington, 1967)

Luxol fast Blue (Disbrey and Rack, 1970)

Periodic Acid Schiffs (PAS) (Culling, 1963)

Phosphotunstic Acid Haematoxylin (Drury and Wallington, 1967)

Toluidine Blue (Disbrey and Rack, 1970)

Victoria Blue (Drury and Wallington, 1967)

<u>Frozens</u>

Acid Phosphatase (Bancroft, 1967)

Cajal Gold Chloride Sublimate for Astrocytes (Drury and Wallington, 1967)

Hortega's Silver Carbonate for Oligodendroglia (Drury and Wallington, 1967)

Hortega's Silver Carbonate for Microglia (Drury and Wallington, 1967)

Hortega's Silver Carbonate for Astrocytes (Drury and Wallington, 1967)

Marchi (Culling, 1963)

Nile Blue Sulphate (Bancroft, 1967)

Osmium Tetroxide - α Naphthylamine (OTAN) (Drury and Wallington, 1967)

Penfield's Combined Oligodendroglia and Microglia (Drury and Wallington, 1967)

Performic Acid Schiff (Bancroft, 1967)

Scharlach R. (Bancroft, 1967)

Sudan Black (Bancroft, 1967).

SECTION 4. QUANTITATION

(a) Reduction Factor

(b) Visual Cortex Depth

(c) Counting and Measuring.

(a) Reduction Factor - Brain tissue shrinks during processing and staining and in view of the comparative aspects of this study the importance of measuring this shrinkage was realised. When blocks were cut using the standard negative method, photographs were taken on a m.m. scale of the faces which contained the visual cortex.

Immediately before counting (to allow for any contraction of the mountant) the stained mounted section was compared with the original block face by using a photographic enlarger.

The negative was placed in a Kodak precision enlarger, and adjusted to give a magnification of x50.8 (1 cm. on negative = 2" on graph paper). The negative of first the block was projected on the graph paper and the outline of the block drawn in. This process was then repeated with the stained section at a similar magnification.

By counting the number of squares within the boundaries drawn to represent the outline of the block or section a measure of the area of the tissue could be obtained, and by dividing the post-processing section result into the block area a measure of the shrinkage or reduction could be obtained.

$$\text{Reduction Factor (R.F.)} = \frac{\text{Original area}}{\text{Post.processing area}} = \frac{\text{AREA 1}}{\text{AREA 2}}$$

e.g. shown in Diagram D. $\text{R.F.} = \frac{498}{307} = 1.62$

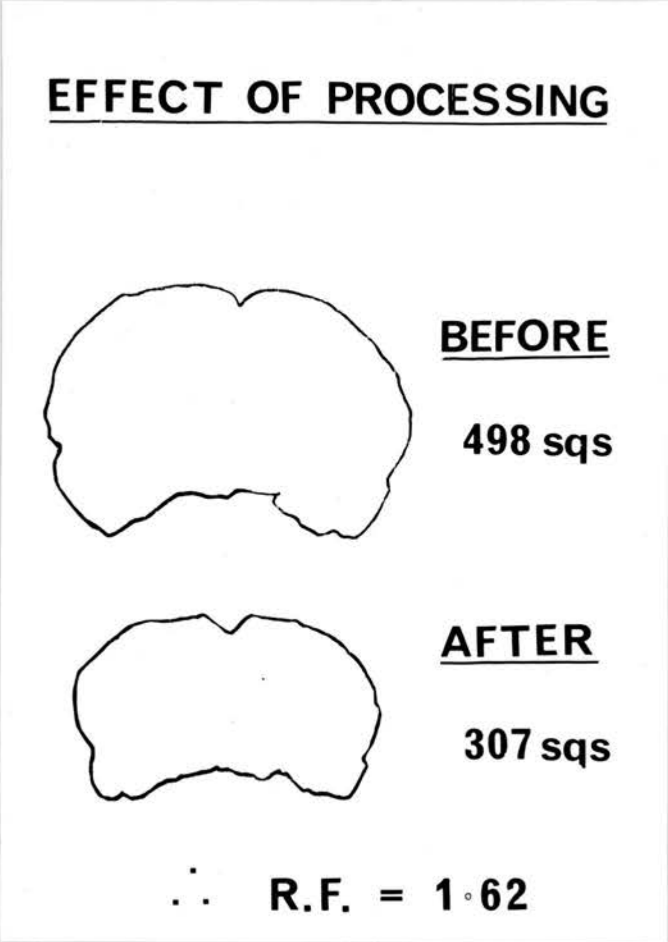


Diagram D.

The reduction factor, obtained by this method is a value for "area" shrinkage, as is shown in the following diagram E, where the lower squares represent microscopic fields. The field with an R.F. of 4 has all the original 16 cells, whereas the field with the R.F. of 2 has only 4 complete cells and some cell fractions. Thus the R.F. affects not only the area visible in a microscope field but also the size of the cells in that field.

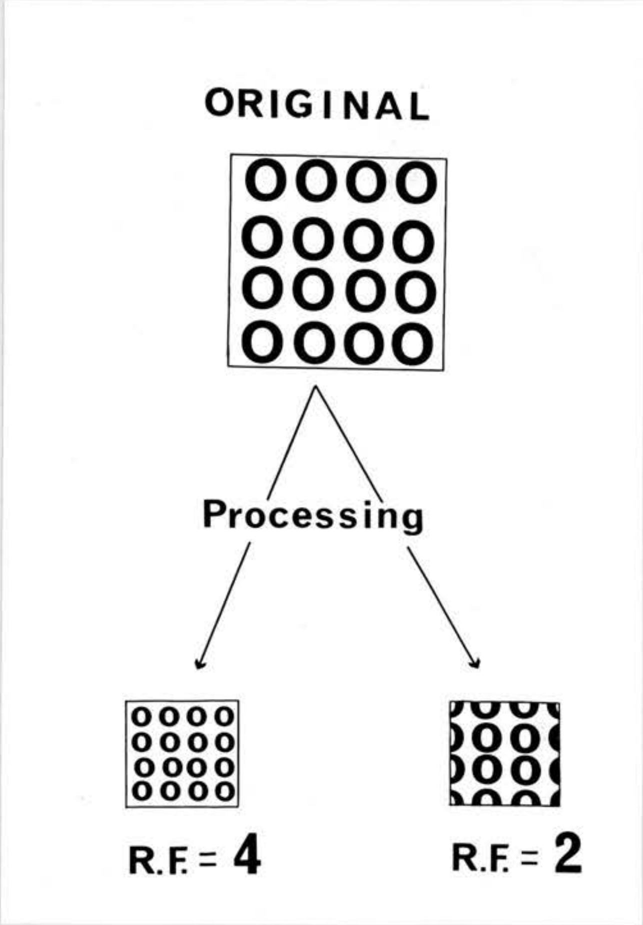


Diagram E.

The reduction factor in addition will affect the section thickness, which is the standard 6µ. unit on the microtome x $\sqrt{RF}$ (the linear reduction factor). Diagram F shows how the standard section from a block which shrinks more during processing will contain a greater volume of the original block than a block which undergoes a lesser degree of shrinkage.

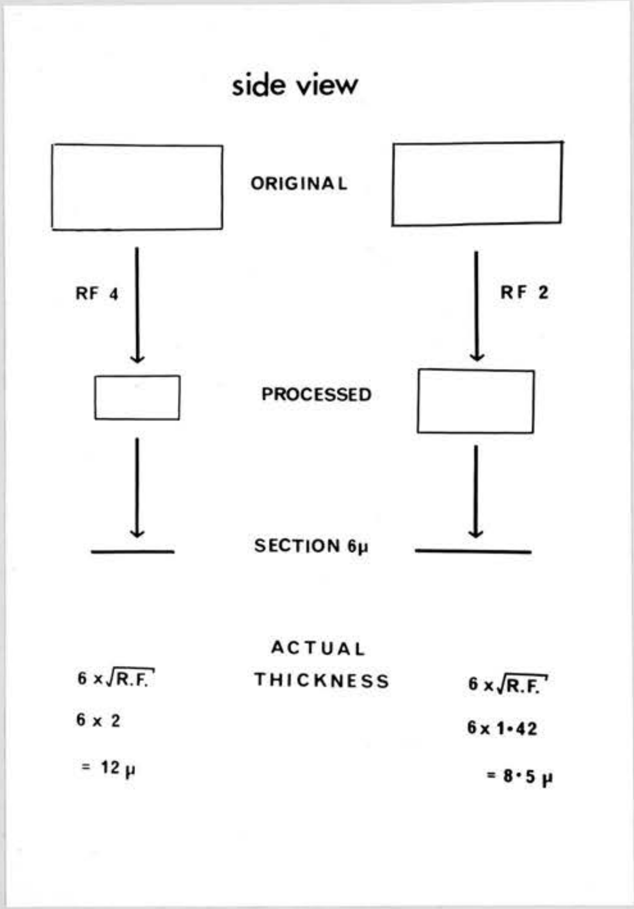


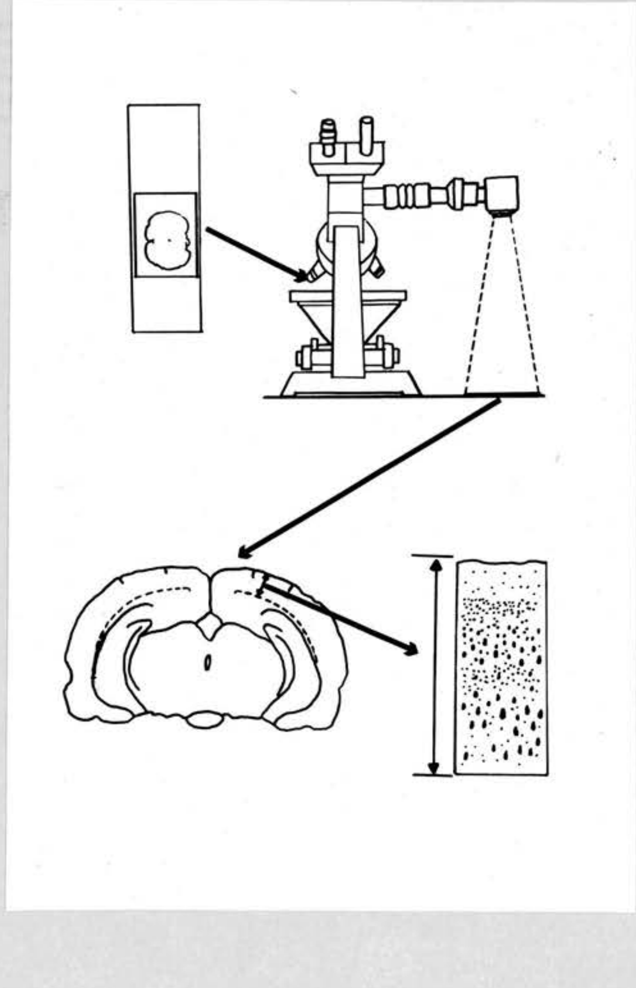
Diagram F.

The sources of error in the method were appreciated and an attempt was made to reduce such errors to the minimum. All camera positions, stop and timing etc. were fixed and a standard developing technique was used. The mm. scale used tended to be the limiting factor, the graduations on the scale were enlarged five times when in the enlarger and thus when localising on the fixed magnification one tried to use the centre of this enlarged graduation. The scale was kept at the same level as the top of the block or slide being photographed.

Another source of error was found in the block of tissue being photographed, namely that the face being photographed was not the tissue actually sectioned. This error was minimised by reducing trimming to a minimum; furthermore, in the rat brain in the area of the visual cortex face this small amount of trimming would not significantly reduce the area of the face.

(b) Visual Cortex Depth - As counting and measuring were performed using an oil immersion lens, the restricted field obtained makes determining the extent of the cortex very difficult. The depth of the visual cortex was measured using a "Wild" microscope (Diagram G) with drawing arm attachment adjusted to give a drawing magnification of x100.

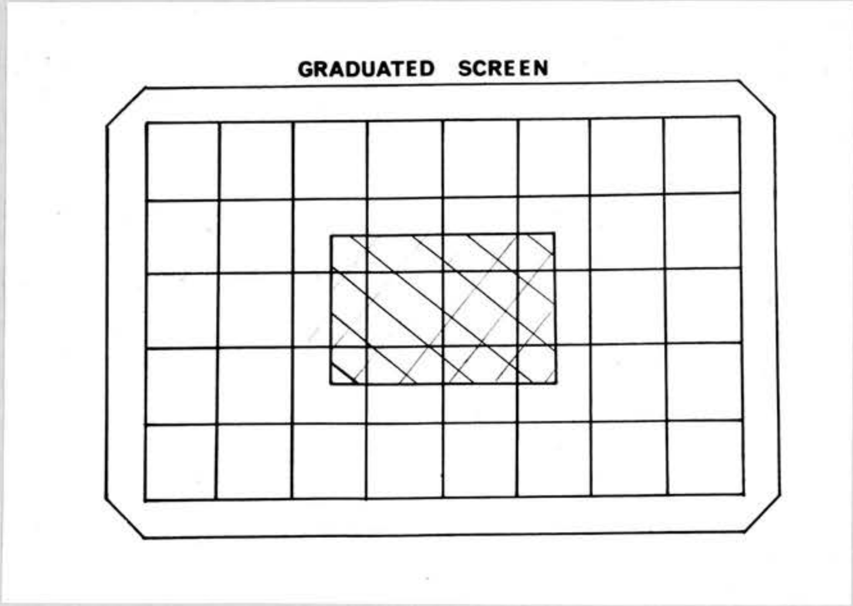
Diagram G.



With this instrument the outer and inner limits of the cortex were drawn over as wide a field as possible. The outer limit of the cortex was taken as the pial surface, and the inner limit between L_6 and the white matter, on the first discernible change from the multiform layer to the rows of oligodendroglia in the white matter. As the overall cortex depth tended to vary due to difficulty in determining the exact lower limit of the innermost layer, five measurements were taken and the average found. By dividing this average width by 100 the "processed depth" could be found. This information was then used to calculate the number of oil immersion fields to traverse the visual cortex from L_1 to L_6.

(c) Counting and Measuring - All quantitative measurements and counts were carried out blind on a "Mikrops" Industrial Projection Microscope. This projected an oil immersion field on a graduated screen at a magnification of x2478. The screen was divided into 1" squares with 1/10" divisions and further marked by 2 rectangles; the larger 8" x 5" and the smaller 3" x 2" as shown crosshatched in Diagram H.

Diagram H.

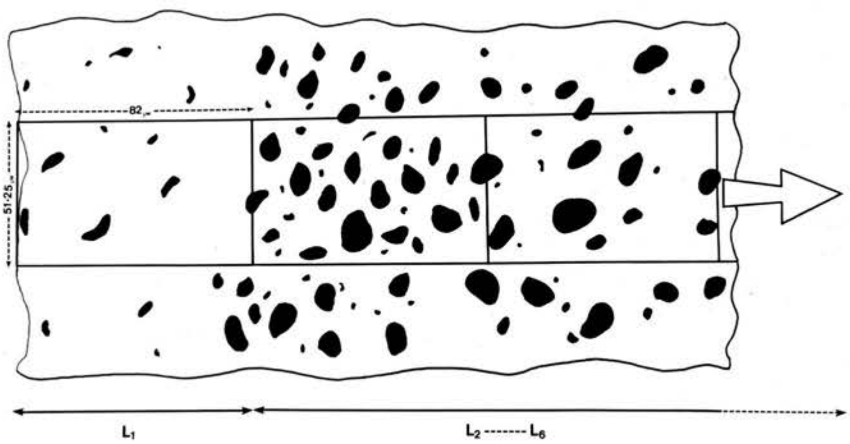


The larger rectangle 8" x 5" was equivalent to a field of $82 \times 51.25 \mu m^2$ on the slide.

Initially an attempt was made to quantitate the visual cortex by taking measurements in the centre of each lamina. However, it was found impossible to demarcate the lamina exactly (L_2 and L_3 for example are merged in the rat cortex) so a system was devised to ignore the lamina and to quantitate the entire depth of the cortex, only separating cell sparse L_1 which is clearly demarcated from the other cell rich laminae.

On the processed section the processed total depth of the visual cortex was known from the measurements obtained by using the "Wild" drawing arm. The field visible on the projection screen was known ($82 \times 51.25\ \mu m^2$), so by division of the total depth by the width of the field ($82\ \mu m$) the number of fields required to traverse the cortex could be found.

Diagram I.



Thus a method was established, where a known volume of the visual cortex could be quantitated by using a series of fields or boxes. The total number of boxes required to traverse the field was not necessarily a whole number, fractions of boxes could also be used. This also applied to the clear demarcation between L_1 and L_2.

The criteria to be examined were cell size and cell density, therefore in every field projected on the screen, the microscopist noted :

(i) The total number of cells in the 8" x 5" field, ignoring those on the upper and left hand side which were touching those borders.

(ii) The dimensions of five cells, those in or nearest to the centre 3" x 2" rectangle. The dimensions of the cells were (a) the length i.e. the longest axis and (b) the width at right angles to the previous measurement.

(iii) Those cells on the left hand side were noted, so that when the stage was moved the next field would start exactly where the previous field had ended.

Gallocyanin stains the perikaryon of the neuron by staining the nucleic acid of the cells, staining both RNA and DNA due to a combination at an acid pH of the phosphoric acid residue of the nucleic acid with the stain (Bancroft, 1967). Gallocyanin also stains glial cells and as with this staining method it is impossible to distinguish between a small neurone and a glial cell, it was decided to measure the perikaryon or cell body of each cell noting the longest axis (length) and longest axis at right angles - width.

The results were recorded (see specimen count, Figs. 8a, b, V.2) for a total of five passages across the visual cortex, each passage consisting of from 10-17 fields. When this data was collected, the resulting information for a known volume of visual cortex consisted of

(i) the number of cells in this volume

(ii) The dimensions of a known sample of these cells.

for L_1 alone, L_2 - L_6 and both combined.

SUMMARY

Perfused Rat → Dissection Brain → Blocking

Blocking → Photographic Negative of Block Face

Blocking → Processing, Cutting and Staining

Processing, Cutting and Staining → Mounted Stained Section

Compare for R.F. → Photographic Negative of Block Face

Compare for R.F. → Mounted Stained Section

Mounted Stained Section → Quantitate

(1) Cortex depth → Volume Tissue Quantitated

(2) Section Thickness → Volume Tissue Quantitated

(3) Cell size)
)
(4) No. of) in Cortex Volume
Cells)

The eyes of every animal were also examined, but could not be quantitated like the visual cortex for two reasons.

(i) No reduction factor for processing could be calculated.

(ii) The retina in the rat is thin, and achieving a thin section cut exactly at 90^{o} to the dorsoventral axis was very difficult. In many cases the minimum possible thickness was not attained and other technical difficulties such as shearing off the retinal layers during processing occurred frequently.

However, the loss of the outer nuclear and rod and cone layers of the Campbell (retinal dystrophic) rat could be shown by using relative values. By means of the "Wild" drawing arm at a constant magnification, drawings of the total retinal thickness could be made. The proportions of this total retinal thickness which comprised the various layers of the retina could then be measured, and the degeneration of the outer nuclear and rods and cones layer could then be plotted on a graph. These results do not have the high level of accuracy of the visual cortex quantitation, but they were useful in that the time scale of the retinal dystrophy could be seen clearly.

SECTION 5. COMPUTOR ANALYSIS AND TREATMENT OF DATA

Due to the large amount of data produced by this method a computor was used for treatment and analysis of the results.

The data sheet was produced as follows, with each microscopic field treated as a "box". (Figs.8a, b, V.2).

S.	Sex. Strain.	Wt.	Age	Date / /	R.F.	Thickness

S.No.	Box No.	Lamina	Cells /Box		a	b	c	d	e	f	g	h	i	j
	1			L										
				W										
	2			L										
				W										
				L										
				W										
				L										
				W										
				L										
				W										
				L										
				W										

L_1 was separated from the rest of the cortex so that results could be produced for

(a) Lamina 1 (L_1)

(b) All other lamina to white matter i.e. L_2 - L_6

(c) Total cortex i.e. sum of above.

The headings shown (with the exception of the date of counting) were coded as follows:

Rat number	Column 1 for experimental E or standard S Column 2-4 for digits
Sex	Male = 1 Female = 2
Strain	Campbell = 1 P.V.G. = 2
Weight	
R.F.	
Thickness (Depth of visual cortex)	

and this information was punched on the first card. This A card thus related to the individual rat's identification.

The B card contained the information on lamina 1 as shown on the data sheet i.e.

(1) The number of boxes or fractions of boxes i.e. the extent of L_1

(2) The total number of cells in these boxes

(3) The dimensions of a sample of the cells in these boxes

and as there were 5 counts, there were 5"B" cards.

The rest of the cards i.e. cards C-G contained similar data on the remainder of the visual cortex.

Thus the basic information for the computer consisted of -

(1) Identification and individual data (on card A)

(2) L_1 - Total area (i.e. no. of boxes)) Card B)

Total no. of cells counted in this area))

Dimensions of a sample of these cells) x 5 as

and size of this sample i.e. number) 5

of cells measured) counts

(3) Rest of the cortex - information as for L_1) Card C - G)

From this information the calculations provided:

(1) Total no. of cells counted for all five data sheets = C

(2) Total no. of boxes for all five data sheets = b

(3) The dimensions of a sample therein

(4) The total numbers of cells measured for this sample = N

This information was subdivided for the following areas:

(A) L_1

(B) $\frac{L_2 - L_6}{2}$ upper part

(C) $\frac{L_2 - L_6}{2}$ lower part

(D) $L_2 - L_6$

(E) Total cortex i.e. $L_1 - L_6$

The sample dimensions of the cells were used to estimate the range of cell sizes present.

Each cell was corrected by using the RF corrected cell area = L x W x RF and located to one of 20 size groups to give a histogram of distribution of sizes.

The mean of all the cells measured was found per animal

$$\frac{\sum L \times W \times RF}{N} = \text{Mean cell size}$$

where N is the total number of cells measured.

As the area counted (no. of boxes x area of box) and the thickness of section "t" are known then the volume counted could be calculated. For this volume the number of cells present "C" is known so the density or number of cells per unit volume could further be calculated.

As explained in Chapter II, to correct the total number of cells present in the section, Murphy's adaptation of Floderus' formula was used.

$$\frac{c}{C} = \frac{t}{t + 2a}$$

where c = Number of cells that should be counted

C = Number of cells that were counted

t = Thickness of section

a = Function of the average cell radius and the factor k

such that $a = \sqrt{r^2 - (\frac{k}{2})^2}$

where r = radius of cell

k = smallest visible fragment.

From the results obtained

$$t = \text{thickness} \times \sqrt{RF}$$

$$a = \sqrt{\frac{\text{mean } L \times W \times RF \times 1.025^2}{4} - \left(\frac{k}{2}\right)^2}$$

k was found to be 1.025μ and thus $\left(\frac{k}{2}\right) = \left(\frac{1.025}{2}\right)$

Corrected number counted c =

$$\left(\frac{t \times \sqrt{RF}}{t \times \sqrt{RF} + 2 \times \sqrt{\text{mean } L \times W \times RF \times 1.025^2 - \left(\frac{1.025}{2}\right)^2}} \right) \times C$$

This number obtained, the corrected number of cells in the section c can be used to find the density, first by finding the area and then the volume of the tissue measured, dividing this into the corrected number of cells c and finding the number of cells per cu.m.m.

i.e. $\frac{\text{corrected no. of cells "c"}}{\text{volume counted in mm}^3}$ = No. of cells per mm^3.

The area calculated was obtained by the formula.

$$\text{Area} = \frac{51.25}{1,000} \times \frac{82}{1,000} \times b \times RF = A \text{ sq.m.m.}$$

where $\frac{51.25}{1,000} \times \frac{82}{1,000}$ = size of a box in m.m.

b = total number of boxes involved

RF = Reduction Factor

and from the area the volume was found by multiplying by the corrected thickness of the section

i.e. $t \times \sqrt{RF}$

Thus volume V

$$V = \frac{51.25}{1,000} \times \frac{82}{1,000} \times b \times RF \times t \times \sqrt{RF} \quad \text{cu.m.m.}$$

where t = thickness of section.

$$\text{Density} = \frac{\text{number of cells counted}}{\text{Volume}}$$

By combining this density result with the frequency distribution of cell size the density of specific cell sizes could be found. e.g. one could compare the density of small cells (< 75 units) for P.V.G. and Campbell rat cortices.

SECTION 6. TECHNIQUE FOR ELECTRON MICROSCOPY

Following perfusion by the method of Schultz and Case (1970), the brain of the rat was dissected out and placed in perfusion solution C. Thin brain slices 1 mm. in width were cut with a razor blade and kept in a layer of solution C. The visual cortex was then cut out of this thin brain slice and further divided to form blocks approx. 1 x 1 mm. x thickness of visual cortex.

The blocks were then immersed in sealed tubes containing 1% osmium tetroxide in stock buffer for one hour at a continuous rotation of 22 revs/min. on a Matburn suspension mixer. After washing in 10% ethanol for 1 - 24 hrs. the tissue blocks were dehydrated in three changes of absolute alcohol, each for 30 mins. Following dehydration the blocks were cleared in propylene oxide for 30 mins. Araldite embedding was the next stage, first at room temperature in araldite mixture for 12 - 24 hrs, then in fresh Araldite mixture at 60°C for 48 hrs.

Araldite Mixture:

(1) ½ lb. Araldite Resin ½ lb. hardener (HY964)

(2) 50 mls. accelerator (DY064) 20 mls. dibutyl phthalate

Mix 19 mls. of (1) with 1 ml. of (2) by continuous rotation for 12 hrs. + at room temperature.

Blocks were sawn from the hardened Araldite and mounted on dowel rods with sealing wax. Sections were cut using a LKB ultra-microtome, 1μ thick sections being used for light microscopy and

80 - 150 nm silver-gold sections for electron microscopy. Thick sections were stained with toluidene blue at 80^{o}C on a hot plate for 1 - 2 minutes, washed and allowed to dry. Thin sections were mounted on Athene 433 grids without a supporting membrane and stained with uranyl acetate, lead citrate as follows:

Uranyl acetate (5ml. of sat. solution in 50% ethanol prepared immediately before use)	10 mins.
10% ethanol wash	20 secs.
Lead citrate (Reynolds, 1963)	2 mins.
Distilled water wash	20 secs.
Allow to dry.	

Sections were kept in petri dishes before examination on an A.E.I. EM6B electron microscope.

SECTION 7. EXPERIMENTAL - THE EFFECT OF DRUGS

The effect of drugs on the visual system other than the eye has rarely been studied, even though some drugs are highly selective, causing pyknosis and lysis of the visual cells and preserving the rest of the retinal layers. A pilot experiment was devised using three drugs :

(i) Iodoacetic acid sodium salt (Sigma)

(ii) 1, 5 - Di - (p- aminophenoxy) pentane dihydrochloride hydrate (M & B 968A)

(iii) 1 - p - Aminophenoxy - 5 - phthalimidopentane (M & B 2948A)

Iodoacetate intravenously is retinotoxic in the rabbit, cat and monkey, 968A orally is retinotoxic in the monkey, dog and cat but not the mouse, rat, rabbit or guinea pig. The status of 2948A was unknown.

The aims of this work were :

(i) To establish a comparable dose rate for the three drugs intraperitoneally.

(ii) To ascertain of these drugs were retinotoxic in the rat.

(iii) To compare the effects of the drugs before and after the visual system was established.

(iv) To follow the development of the lesions, if any, in the eye with the ophthalmoscope.

(v) To kill the rats at various stages and demonstrate the development of lesions, if any, histologically.

Initially to gain some experience in retinal toxicity, two drugs (968A and 2948A) were injected intravenously into rabbits at a dose rate of 10 mgm/Kg. The eyes of these animals were examined ophthalmoscopically for 3 weeks and then the rabbits were killed. Histological sections of eyes were prepared and examined.

This dose rate (10 mgm/Kg.) was also used on rats, intraperitoneally at 1 day of age and adults for both 968A and 2948A. No appreciable effect was noted at this dose rate, so it was decided to increase the dosage to 20 mgm/Kg. The early dose rate of 60 mgm/Kg. of sodium iodoacetate proved fatal, so this was reduced similarly to 20 mgm/Kg.

Formulation of Solutions

	Mol. wt.
1, 5 - Di - (p- aminophenoxy) pentane dihydrochloride hydrate (M&B 968A)	381.9
1 - p - Aminophenoxy - 5 - phthalimidopentane (M&B 2948A)	324.4
Sodium Iodoacetate (NaI)	208.

From the dose rate the strength of solution was calculated, which provided an acceptable volume for injection.

	Molarity	Strength	Dose Rate	Injection Rate	Expt. Animal
968A.	0.05	0.019095g/ml	10 mgm/Kg	0.5µl/g	Rabbit
	0.005	0.0019095g/ml	10 mgm/Kg	5µl/g	Rat
	0.01	0.0038190g/ml	20 mgm/Kg	5µl/g	Rat
2948A.	0.05M	0.01622g/ml	10 mgm/Kg	0.5µl/g	Rabbit
	0.005	0.001622g/ml	10 mgm/Kg	5µl/g	Rat
	0.01	0.003244g/ml	20 mgm/Kg	5µl/g	Rat
NaI.	0.15M	0.00312g/ml	62.4 mgm/Kg	20µl/g	Rat
	0.1M	0.0208g/ml	62.4 mgm/Kg	3µl/g	Rat
	0.19M	0.004g/ml	20 mgm/Kg	5µl/g	Rat

Both 968A and sodium iodoacetate are soluble in water and no problems were associated with the production of these solutions. However, 2948A is a free base and only 0.1% soluble in water, so the acid base had to be produced to make the compound soluble. This was achieved by dissolving the desired weight of 2948A in the minimum amount of glacial acetic acid (Analar. M.wt = 60.05) over ice, usually about 0.2 ml. of acid, which was then diluted with water to 5 mls. A control solution was made up of a similar strength of acetic acid to cover the possibility of complications due to the acid. Distilled water was used as control for 968A and sodium iodoacetate.

Site of Injection The rabbits were injected intravenously in the ear vein. This route of injection was not used in the rats, as one of the principal aims of this work was to administer the drug before the visual system was mature. This involved injecting 1 day old rats, and the intraperitoneal route was chosen for ease of administration.

<u>Time on Test</u> The question of experiments on animals has raised a great deal of public disquiet, and particularly those involving the sight of laboratory animals. In this highly sensitive area it was decided to limit the time on test to four weeks, except for the case where two animals were allowed to survive up to 8 weeks. During the test the animals were examined twice daily during weekdays and once daily during weekends, to ensure that the rats were suffering no ill effects. In practice, the effect of the drugs on the visual system was minimal and those animals which survived the injections showed no visual defects at all - ophthalmoscopic examinations during the rat tests were negative. The test rats were sacrificed at weekly intervals during the experiment, the tissues of younger animals (1-2 weeks old) where perfusions were difficult being fixed by immersion, and the older animals by perfusion.

CHAPTER IV

THE EYE

		Page
SECTION 1.	OPHTHALMOSCOPY	
	(a) P.V.G.	111
	(b) Campbell	111
	(c) Rabbit	112
SECTION 2.	HISTOLOGY	
	(a) P.V.G.	113
	(b) Campbell	114
	(c) Rabbit	116
SECTION 3.	QUANTITATION	118
SECTION 4.	SUMMARY OF RESULTS	121

CHAPTER IV

SECTION 1. OPHTHALMOSCOPY

Ophthalmoscopy was performed on experimental animals at regular intervals during the test and on all animals before perfusion.

The fundus of the rat eye is first visible at 21 days in both P.V.G. and Campbell strains, and can be examined throughout life although cataract development makes ophthalmoscopy very difficult in the Campbells, where cataract can develop at any age from 3 months.

The remnants of the hyaloid vessels can be seen in some animals up to the age of 8 weeks, and occasionally in older rats. Suture lines in the lens are visible over much the same period. Keratitis and traumatic lesions on the cornea were much more frequent in the Campbell rat.

The vasculature of the retina in the rat consists of six main arteries, six main veins with superficial, intermediate and deep capillary networks (Cairns, 1959), or six main arteries and four vorticose veins (Janes and Bounds, 1955). However, in both strains of rats used in this work considerable variation in the number of both arteries and veins, the arteries ranging from 6 to 8 and the veins from 4 to 6, can be seen, as in Diag. A.

These vessels radiate out from the optic disc, which often consisted of a small white irregular area surrounded by vessels. There is no disc or macula as recognised in the human.

(a) P.V.G. - The vessels in the P.V.G. at the disc had a 3D effect with the small arteries appearing above the veins, i.e. nearer the observer when viewed ophthalmoscopically. The veins are broader and darker than the arteries, and both were clearly visible on a white glistening background.

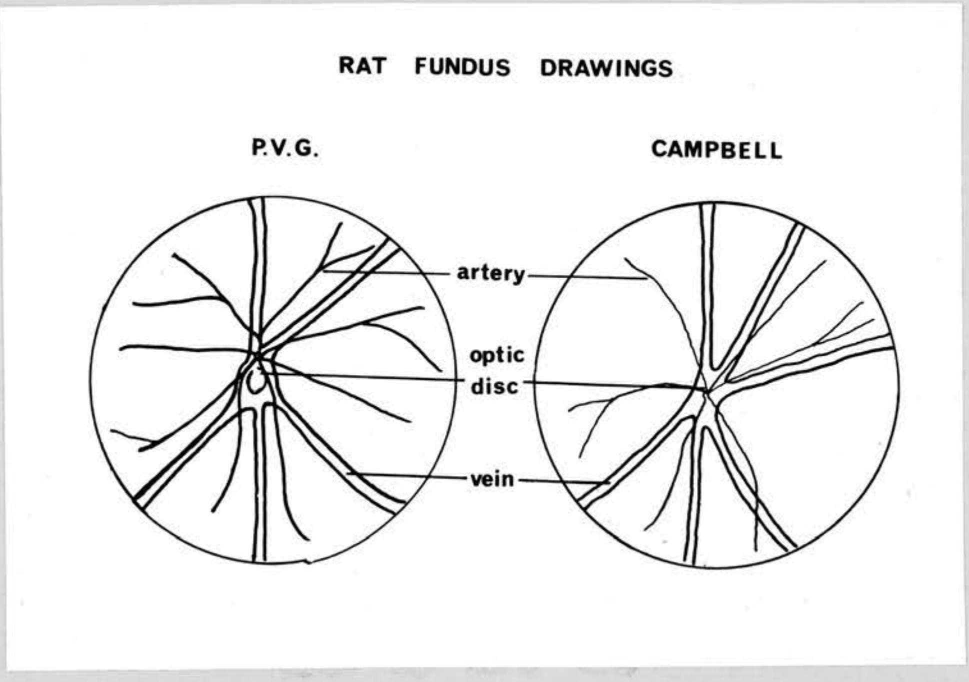


Diagram A.

(b) Campbell - The Campbell fundus invariably seemed more difficult to get in perfect focus. The fundus appeared pink just out of focus, but when sharpened, it was identical in colour to the P.V.G. The 3D effect of veins and arteries at the optic disc was lost, and the arteries appeared thinner in the Campbell. The veins were thinner and flatter, with the loss of the sharp outline found in the P.V.G.

The differences were seen clearly at 60 days, and although some changes could probably be seen before this date a high degree or expertise would be required to notice them.

No ophthalmological signs at all were observed in the rats treated with retinotoxic drugs.

(c) Rabbit - The rabbit fundus appears much more vascular than the rat and much more easily seen. The optic disc is positioned above the posterior pole of the globe. The arteries and veins which enter and leave at the optic disc occupy much of the fundus as can be seen in Diagram B.

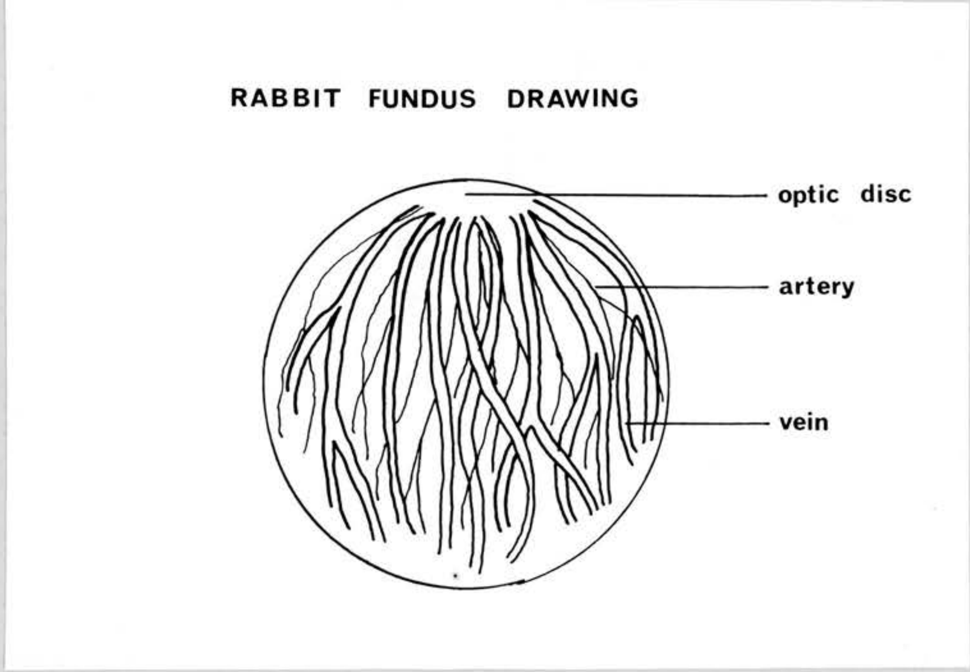


Diagram B.

Two rabbits which were given 2948A and 968A intravenously failed to show any clinical signs. Ophthalmologically 968A produced some oedema and loss of translucency over the 3 week period; whereas 2948A only produced a slight oedema.

SECTION 2. HISTOLOGY

Sections of eye were examined in every animal perfused. The development of the normal P.V.G. and the dystrophic Campbell eye was compared to find a time scale for the degeneration, which could then be compared with changes in the visual cortex.

(a) Normal P.V.G. Development

Day 1: The inner nuclear and outer nuclear layers of the retina are combined as a single broad band lying on the pigment epithelium. The inner plexiform layer separates this combined nuclear layer from the ganglion cell layer. Melanin is present at this stage in the uvea (choroid, ciliary body and iris) and the pigment epithelium is in the form of short rods and dots. The hyaloid vessel system is present and starts to involute at 7 days. The eyelids are closed at birth and open at about 2 weeks of age.

Day 5: Early separation of the inner and outer nuclear layers is visible at this stage. Protrusion of segments from the outer nuclear layer tends to separate this nuclear layer from the pigment epithelium layer. More melanin is present in the uvea and pigment epithelium.

Day 7: Further separation of the nuclear layers and further extrusions of the rods from the outer nuclear layer can now be seen. The hyaloid system is present although it starts to involute at this stage.

Day 10: Early differentiation of the rods into inner and outer segments, the former eosinophilic and the latter now staining with Haematoxylin and Eosin (Fig. 3a, V.2)

Day 14: The rats eyelids separate.

Day 14 - 30: Further development of the rods and cones layer with differentiation of the outer nuclear layer. The retina has more or less attained adult histology by day 30 (as can be seen from Figs. 3b, c., V.2)

Age Changes.

The eyes of rats from 400 - 500 days of age were examined. The P.V.G. showed an occasional lens opacity visible with the ophthalmoscope but as the processing technique required the loss of the lens this was not confirmed histologically.

In some rats Bruch's membrane (between pigment epithelium and chorio capillaries) was more prominent and occasionally showed some hyaline change. With Periodic Acid Schiff (PAS) staining clumps of PAS +ve material or debris could be seen at the junction of the inner and outer segments of the rods, presumably from breakdown of the rods, although the pigment epithelium did not show any signs of proliferation or phagocytosis.

(b) Development Campbell Retina (Figs.3d, e,f,V.2)

Day 1: At birth the Campbell retina is similar to the P.V.G. in that the inner and outer nuclear layers are combined. However, unlike the P.V.G. the Campbell uvea and pigment epithelium layer is unpigmented.

Day 2 - 14: Development as for the P.V.G. (Figs.3d, 4b, V.2). From these photographs it would seem that the 10 day old Campbell retina is thicker than the P.V.G. retina. This is because of the plane of section, which is not perpendicular to the layers in the Campbell as it is in the P.V.G. i.e. the minimum thickness has not been attained. However, the various layers of the retina are present in the same proportion in both eyes, e.g. the outer nuclear layer in Figs. 4a, b, V.2 accounts for 33% of the total retina.

Day 15: The outer segments in the Campbell rods and cones layer is larger than the inner segments at this stage, unlike the P.V.G. where the inner segments are equal in width to the outer.

Day 17: Early degeneration and vacuolation in the outer segments.

Day 18 - 24: This degeneration continues with breakdown of the rods and cones layer (Fig. 3e, V.2).

Day 25: The rods and cones layer loses its characteristic staining with H & E, i.e. eosinophilic inner segments and non-staining outer segments.

Day 26 - 50: The degeneration of the rods and cones and outer nuclear layer continues with lysis of these layers. Occasionally a few pigment epithelium cells appear to have migrated into this degenerating layer, and the pigment epithelium layer cytoplasm often includes large vacuoles (Fig. 3f, 4d, V.2).

Day 50 - 70: Pyknosis and lysis of both the outer nuclear and rods and cones layers is almost complete. Some hyalinisation of Bruch's membrane and proliferation of glial cells. Detachment of retina during processing at the pigment epithelium/choroid and not through the rods and cones layer as is usual (Fig.2b, V.2).

Age Changes.

Day 400/500: The aged Campbell retina is fibrosed and distorted with loss of the regular layer structure. The retinal blood vessels are thickwalled, distended and run irregularly throughout the retina.

Retinal detachment due to processing tends to occur at the pigment epithelium/choroid junction and not as is usual through the rods and cones layer. Some degeneration is shown by the presence of Periodic Acid Schiff +ve material throughout the retina. Occasional intraocular haemorrhages can be seen.

(c) Rabbit Eye Histology

The normal rabbit retina is composed of the usual 10 layers. The rabbit is like the rat in that it has a rod dominated retina. Muller's fibres are very prominent in the rabbit. The ganglion cells vary in size and some of their number are multinucleated.

The controls used were pink eyed so no pigment was present in the uvea or pigment epithelium.

Drug Treated Rabbit Eye Histology

968A. 1, 5 - Di - (p- aminophenoxy) pentane dihydrochloride hydrate

Artefact detachment of the retina during processing makes histopathology difficult but arcade formation involving the outer nuclear and rod and cone layers is visible. This arcade formation is probably due to swelling and degeneration in the rods and cones layer with proliferation and migration of the phagocytic pigment epithelium cells. This degeneration is patchy in distribution but affects principally the outer segments. The ganglion and outer nuclear layers appear normal.

<u>2948A</u>. <u>1 - p - Aminophenoxy - 5 - phthalimidopentane</u>

This drug appeared to have very little effect. An occasional small arcade is visible but otherwise the retina appears normal.

SECTION 3. QUANTITATION

As explained in Chapter II, no allowance for processing reduction (R.F.) was made for the eye sections because of technical difficulties. Eyes are difficult to process and retinal detachment is a constant problem, as is blocking the eye to get the minimum thickness of retina (as seen in Figs. 3, 4, V.2). However quantitation of the P.V.G. and Campbell eyes was thought desirable if only to get some idea of the time scale of the development and dystrophy of the retina.

Comparable values were obtained by measuring the actual thickness of the various layers and the total retina using the Wild drawing arm attachment, and converting these results into percentage values. Thus regardless of processing, values of width of various layers can be obtained. However, these values are dependent on total retina thickness, so the first step is to take the actual values obtained, and disregarding all the objections outlined above, draw graphs of total retinal thickness against age (Fig. 5. Table A,V.2). From this graph, despite the wide variations in results, it can be seen that after the first 30 days the Campbell retina becomes thinner than the P.V.G. This complements the histological findings where loss of the outer nuclear and rod and cone layers takes place from day 30 to 60. Furthermore, it can be seen that after day 50 the Campbell retina attains a moderately constant thickness. The P.V.G. retina from the graph, attains adult dimensions by day 80. This means that the percentage readings will be influenced by these variations in total retinal thickness.

The graph showing the width of the outer nuclear layer (Fig. 6: Table A, V.2) in percentage of total retina shows clearly in the Campbell the retinal dystrophy, where loss of this layer occurs over day 30 - 80. The P.V.G. maintains a reasonably constant outer nuclear width percentage, despite the fluctuations in total retinal thickness.

This constant percentage is also shown in the P.V.G. inner nuclear width (Fig.7: Table A, V.2). The Campbell inner nuclear layer, however, fluctuates - is this due to the overall shrinkage of the retina or is it due to variation within the inner nuclear layer? If the inner nuclear layer remained at a constant value as it does in the P.V.G., then as the total retina shrinks with age, the various layers must be thinned by a comparable amount. In the Campbell, however, from day 30 there is a relatively rapid loss of two layers, i.e. the outer nuclear and rods and cones layers. The retinal shrinkage produced by this loss is not matched by a comparable thinning in the inner nuclear layer so the percentage contribution of this layer rises as seen on the graph.

<u>Conclusion.</u> A time scaled diagram of the histology and quantitation of the Campbell dystrophic retina can be drawn up. From this diagram it can be seen that histology reveals the earliest change in the Campbell retina, and shows the time lag between the early degeneration and the retinal shrinkage which can be measured quantitatively. Similarly, the time lag between the dystrophy starting and the ophthalmoscopic changes is also revealed.

SECTION 4

Age in Days	P.V.G.	Campbell Histology	Campbell ophthalmoscope	Campbell Quantitation
1 - 10	Inner nuclear + outer nuclear layers combined. Separation IN + ON layers. Early diff. of rods.	Normal development.		
11 - 20	Eyelids open	First diff. IS/OS of rods. Early deg. in R & C layer.		Normal development
21 - 30	Eye fully formed.	and involving ON	Fundus visible	
31 - 40				Retina
41 - 50		Degeneration of ON and R & C layers		Shrinkage
51 - 60			Ophthalmic changes in vasculature	Loss of ON + R & C layers
61 - 70				
71 - 80	Eye attains adult dimensions.			

IN = Inner nuclear
ON = Outer nuclear
IS/OS = Inner segment/Outer segment
R & C = Rods and cones

CHAPTER V

THE VISUAL CORTEX

Page

SECTION 1. HISTOLOGY OF THE VISUAL CORTEX

(a) Neurone 123

(b) Neuroglia 125

(c) Visual Cortex
- Lamination 129
- Development 131

SECTION 2. QUANTITATION OF VISUAL CORTEX

(a) Computer Treatment 133

(b) Individual Histograms of Cell Population 135

(c) Mean Cell Size 136

(d) Cell Density 137

(e) Cortex Depth 139

(f) Density of Cells of <75 and >75 units 140

(g) Density of Cell Groups of <25 units 141

(h) Density of Cells of <100 and >100 units 141

(i) Density of cells of 100-<200 and >200 units 142

(j) Summary 142

SECTION 3. MISCELLANEOUS QUANTITATION

(a) Fixation 144

(b) Reduction Factor 144

(c) Drugs 145

SECTION 4. STATISTICS

(a) Introduction and Method 146

(b) Summary of Significance 148

SECTION 5. SUMMARY 153

CHAPTER V

SECTION 1. HISTOLOGY OF THE VISUAL CORTEX

Over many years, numerous disciplines have used a wide variety of techniques to study the cerebral cortex. In histology alone there is a multiplicity of special stains for various constituents of the cortex, which although admirable for their purpose often fail to stain the other components of the tissue. Within the confines of this quantitative work, it was decided to use one standardised stain, Gallocyanin, for the quantitation and to use electron microscopy and special stains for further investigation.

Gallocyanin stains nucleic acids, in neurones, glia and endothelial cells of the cortex. The RNA of the nucleolus and cytoplasm and the nucleic DNA are all stained blue on a colourless background as axons, dendrites and glial processes fail to stain.

(a) Neurones

The perikaryon or cell body of the neurone consists of the nucleus, nucleolus and cytoplasm but not the axon or dendrites. The neurone perikaryon of the cerebral cortex shows a wide variety of shapes and sizes, but basically two types of neurones can be recognised. These are firstly the conical shaped pyramidal neurone with its long axis at right angles to the pial surface and, secondly, the almost spherical stellate neurone. Other shapes can be seen in the cortex, e.g. fusiform cells, but these are thought to be variations on the basic stellate or pyramidal types (Sholl, 1967). Much of the work on classification of neurone types has utilised the Golgi stain, but these different types of neurone can also be recognised in Nissl stains, such as Gallocyanin.

In Gallocyanin stained sections (Fig. 7, V.2) the nucleus of the neurone is large, round and vesicular with a distinct nuclear membrane, and depending on the plane of section, a nucleolus. The cytoplasm contains various amounts and patterns of Nissl bodies, and the cell membrane stains distinctly, except at the junction of cell body and the axon or dendrites of which only an occasional ghost outline remains. Nerve cells are highly sensitive to alterations in their environment, (Cappell and Anderson, 1971), and as no mitotic division of neurones occurs after the neuroblast stage, dead neurones are not replaced.

The possible changes in a neurone which are degenerative in nature are swelling, shrinkage, vacuolation, pigment changes, fibrillary degeneration and chromatoylsis. Primary degenerations (produced by agents acting directly on the cell body e.g. vascular disease, poisons) are either acute or chronic. ~~secondary.~~ Acute change is characterised by swelling of the cell with diminution and loss of Nissl substance - changes which correspond to central chromatolysis. Recovery may or may not occur, depending on the cause. Chronic changes usually result in shrinkage of the cell, often with loss of fine structure, reduction in density of Nissl substance and vacuolation which may ultimately lead to death of the cell.

Secondary degeneration is produced in the neurone as a result of damage elsewhere e.g. Wallerian degeneration of the axon, which if severe can lead to central chromatolysis of the neurone. Another form of secondary degeneration is transneuronal atrophy (seen in the visual system, where the cells of the lateral geniculate body atrophy after lesions of the optic nerve) where the principal afferent connections have been destroyed.

(b) Neuroglia

The glial elements of the central nervous system can be classified as macroglia, microglia and ependyma. The macroglia (astrocytes and oligodendroglia) and ependyma are derived from ectoderm, whereas the microglia are believed to be derived from mesoderm.

Oligodendroglia - These are the smallest of the macroglial cells, and are found in both grey and white matter. In the white matter these cells occur as interfascicular glia, and in grey matter as perineuronal satellites and occasionally around blood vessels. The visual cortex oligodendroglia are small with a round or oval nucleus, usually darkly staining although lighter staining forms can be found. The nuclear chromatin is in the form of darkly staining clumps.

Oligodendroglia by hypertrophy of the cytoplasm can exhibit acute swelling, for example in acute toxic conditions. The cell may recover or show degenerative pyknotic changes leading to dissolution of the cell.Hyperplasia of oligodendroglia occurs in toxic and inflammatory conditions, e.g. satellitosis around degenerating neurones.

Astrocytes - These cells are divided into protoplasmic and fibrous types. The protoplasmic astrocyte is found in grey matter only whereas fibrous astrocytes are found throughout the nervous system. In the cortex very few fibres are present in L_2 and L_6 and practically none in L_3, L_4 and L_5 (Greenfield, 1958). A typical astrocyte is about a third larger than an oligodendroglia. The large oval pale nucleus contains fine sparse chromatin unlike the oligodendroglia where it is clumped and dense. A small nucleolus can be seen depending on the plane of section.

Astrocytes resemble fibroblasts in their reactions. Reactive changes such as hypertrophy and hyperplasia, with production of glial fibrils, called gliosis, can be seen after damage to the nervous system. Astrocytes are less vulnerable than neurones to noxious processes, but where the process is severe e.g. infarct, these cells undergo regressive changes leading to necrosis. Two different types of reactive astrocytes, the Type I and Type II Alzheimer have been described (Cavanagh and Kyn, 1971 a and b).

Ependyma - These cells line the ventricles and are not usually involved in diseases of the visual cortex.

Microglia - These small cells are distributed throughout the nervous system, although they are more abundant in grey matter. In the resting or "fixed" state only the nucleus is visible in Nissl stains, and can be found free or as perineuronal or perivascular satellites. The nucleus is small, hyperchromic and irregular in shape - oval, rounded, curved, triangular or polyhedral.

Microglia play an important part in central nervous system disease, when these cells, normally quiescent, undergo hypertrophy and hyperplasia. They exhibit phagocytosis e.g. in degenerative conditions, after which they slowly make their way to the walls of blood vessels where they accumulate both round and within the perivascular space. Phagocytosis of effete neurones (neuronophagia) is another function of microglia.

The other cell found in the visual cortex is the endothelial cell of the capillaries from the middle cerebral artery which supplies the greater part of the cerebral hemisphere. The various lamina differ markedly in the amount of vessels per unit volume of tissue, but overall there is no change in vascularity between blind and sighted rats in the striate area (Zeman and Innes, 1963). Endothelial cells can be recognised by their curved fusiform shape and by the presence of the blood vessel. Pericytes with a crescentic nucleus can also be found in the cortex wrapped around the capillaries but outside the basement membrane (Figs. 28a, b, V.2).

With Gallocyanin stained sections, classical examples of these cells can be identified principally on size and nuclear characteristics. However, it was found impossible to identify every cell with certainty so no attempt was made during the quantitation to classify the cell types.
(Greenfield et al, 1958; Anderson, 1966; Jubb and Kennedy, 1970; Cappell and Anderson, 1971; Ling et al, 1973; Cavanagh, 1970).

	ASTROCYTES Protoplasmic Fibrous	OLIGODENDROGLIA	MICROGLIA "Fixed"	 Active
Cell Size	Largest	Small	Smallest	Enlarged & spherical
Nucleus	Large, ovoid Pale Fine sparse chromatin	1/3rd smaller than astrocyte Round or oval Pale - Dark Dense clumps chromatin	Narrow elongated Hyperchromatic Even distribution chromatin	Elongates:irregular
Cytoplasm			Scanty	Varies - becomes visible with routine stains (H&E) (basophilic)
Processes	Short Numerous Many branches / Long Slender Few branches	Few short delicate	Short irregular	May retract
Function	Support: Nutrition	Nutrition: myelin formation	Phagocytosis	Phagocytosis
Homologue	Fibroblast		C. T. histiocyte	

(c) Visual Cortex

Lamination - Microscopy of the visual cortex of the rat reveals a stratified appearance due to the differences in density of the cells just described throughout the cortex (Fig.7,V.2). The lamination of the cortex has been a controversial subject for many years simply because qualitative criteria are used to differentiate the layers. The six layered cortex in every current textbook is (sic) "but little better than arbitrary and conventional" (Economo in Sholl, 1967).

Lamination, however, is useful for descriptions of the cortex,using the basic 6 layers except for L_2 and L_3 which are merged in the rat. These layers are :

L_1 - Molecular or plexiform layer

L_2- L_3 - External granular and external pyramidal layer

L_4 - Internal granular layer

L_5 - Internal pyramidal or ganglionic layer

L_6 - Multiform or fusiform cell layer.

These layers can be seen in Gallocyanin stained sections. However, with the axons and dendrites unstained it is more difficult to distinguish neurone types, although shapes can be recognised.

L_1 - This cell sparse area is clearly seen extending from the pial surface to the cell rich L_2 L_3. This layer contains many microglia and endothelial cells but no neurones. L_1 is rich in fibres which accounts for it being called the plexiform lamina.

L_2 L_3 - These layers extend from the sharp demarcation of L_1 and merge into L_4, this being recognised by the transition into the higher cell density of L_4. The cells present are usually round, oval or pyramidal in shape, and although the sizes of these cells vary, none are as large as the big cells of L_5; these layers are granular in appearance, and contain pyramidal cells which accounts for the names.

L_4 - This layer is of a higher cell density than the layers on either side, into which it merges. The borders are not distinct but a rough indication of the width of this layer shows it is much narrower than either L_2-L_3 or L_5. The cells are mainly round and small and are said to be darker staining, although with the standard staining routine used in this work this was difficult to confirm. Once again this layer is granular in appearance.

L_5 - This layer is wider than L_4 and can be recognised by the low density of cells. Again the borders merge into the layers on either side. L_5 contains the largest cells in the visual cortex, broad pyramidal cells with approximately equal sides. Small pyramidal and round cells, in addition to microglia and capillaries are also present.

L_6 - The multiform layer contains mainly oval or spindle shaped cells which have their long axes parallel to the pial surface. The cells again vary in size and shape. The indistinct junction with L_5 can be recognised by the change from the low cell density of L_5 to the higher density of L_6. L_6 ends at the white matter, and in this work the lower border was taken as the change from the multiform layer to the horizontal rows of interfascicular glia in the white matter.

This description reveals the fault in using qualitative criteria - what size is large or small and what is the difference between wide and narrow? In addition the lack of easily defined borders and the inherent variability of the strata makes any attempt to compare the widths of each of the layers very difficult.

This description of the visual cortex applies to both strains of rat; no difference in qualitative histology could be found. The solution was to proceed to quantitation to see if any difference could be found.

Development of cerebral cortex - The rat is born with the visual cortex immature, consisting of tightly packed undifferentiated cells. Neuroblasts, which form in the neuro-epithelial layer along the ventricle migrate through the previously formed neuroblast layers to occupy a more superficial position (Angevine and Sidman, 1961). This means that the first neuroblasts formed develop into the infragranular layers (L_5 and L_6), and those formed later will become the granular and supragranular layers (L_4, L_2-L_3). Thus development starts from the innermost layers and works up to the outermost layers.

At birth the rat cortex of both P.V.G. and Campbell rats consists of closely packed undifferentiated cells, the density of which is higher towards the pial surface. L_1 can be distinguished clearly, and below this are found columns of chromophilic undifferentiated cells. L_5 can be recognised as a thin band of larger cells with the multiform L_6 below.

On day 5 the cortex has lost some of the orderly columns in the outer cortex, although the cells in the outer cortex are still undifferentiated, closely packed and darkly staining. Large pyramidal cells are visible in L_5 and between L_2-L_3 and L_4. By day 10 all the layers can be distinguished although it is difficult to distinguish between L_2-L_3 and L_4, because the cells in this region are still quite closely packed. The cells are starting to lose their chromophilia.

By day 20 the lamina are clearly marked and the changes from this stage are quantitative rather than qualitative. Again no difference was discernible qualitatively between the Campbell and P.V.G. development.

SECTION 2. QUANTITATION OF THE VISUAL CORTEX

As described in the Materials and Methods chapter, a quantitative system was devised to measure the perikaryon size and density throughout the visual cortex of normal and blind rats. Correction factors to allow for variations in processing and counting were used to ensure comparability of the results.

(a) Computer Treatment

The results obtained from the projection microscope were noted on data sheets (Figs. 8a, b, V.2) and this data was punched on computer cards. A programme was devised such that the computer produced a print out for each individual animal (Specimen print outs, Figs. 9a, b, V.2). The first line of the print out comprised the identification of the animal i.e. sex, strain, age, weight and reduction factor. The "processed" thickness of visual cortex in μm is given as is the "corrected" cortex thickness i.e. "processed" thickness x $\sqrt{RF}$.

The two tables on the print out (Tables A & B, Figs.9a,b,V.2) are concerned with cell size and density. Table A lists :-

(α) Number - the number of cells in the volume of cortex counted.

(β) Mean size - the volume of cortex which was quantitated.

(γ) Correction Factor - this is to allow for split cells which are counted as whole cells by the microscopist.

(δ) Corrected Number - the number of cells in the volume quantitated after correction for split cells.

(ε) Corrected density - By dividing the corrected number of cells in a known volume by the volume the density of cells in a standard volume (1 mm^3) can be calculated.

For each of these results there are five columns. These columns are an attempt to subdivide the cortex, not into lamina but into fractions. The first column, i.e. the left hand side, refers solely to L_1 which is easily and clearly separated from the rest of the cortex. The second and third columns are respectively the upper and lower halves of the rest of the cortex $L_2 - L_6$, i.e. $\frac{L_2 - L_6}{2}$ The cortex $L_2 - L_6$ is represented by column 4 and the total cortex i.e. $L_1 - L_6$ is shown in the last column.

Table B of the print out refers to the size distribution of cells within the cortex, in effect a histogram. From the range of sizes in the sample and the corrected density, the number of cells of different sizes present in unit volume can be calculated. Again five columns are used to split the cortex.

The cell sizes were measured in arbitrary units and corrected for processing by multiplying by the R.F. ("l" x "w" x RF) where "l" and "w" are the two maximum axes at right angles to one another. As no attempt was made to use π at this stage the results are in arbitrary unitsx. These arbitrary units were also used in Table B to provide data for the histograms dividing the results into 20 groups, the first group giving the number of cells between 0 - <25 arbitrary units, the second those between 25 - <50 and so on.

The results from the computer print outs have been summarized in table form at the end of Volume 2. These results are arranged in sex and strain order (1 - 4) and as group and individual tables (Tables B and C).

(b) <u>Individual Histograms of Cell Population</u>

From the results in Table B of the print out individual histograms of cell population can be produced (Fig. 10, V.2). The histograms selected for this figure show the two strains at 3 ages, i.e. 30, 70 and 150 days of age. Individual variation will contribute to these histograms but it can be seen that at 30 days there appears to be little difference between the strains. By 70 days however, the P.V.G. shows a greater frequency of larger cells i.e. those cells (>200 arbitrary units) and a smaller frequency of smaller cells. This finding is duplicated at 150 days. Thus the conclusion is that at sometime between 30 and 70 days of age some change occurs separating the P.V.G. from the Campbell.

The question then arises - is this conclusion simply due to individual variation or does this difference occur throughout the age range after 30 days of age? The individual histograms are useful in that the cell population can be represented simply, but analysis of all the histograms would be very difficult. In fact one of the problems of this work was reducing the masses of data which accumulate for an individual to a single value which then could be compared throughout the range.

The obvious solution with the cell population histogram was to use the mean cell size value, as shown by the arrow in the figure.

(c) Mean Cell Size

This result can be found by finding the sum of the cell sizes measured, and dividing this by the number of the cells measured in the sample.

$$\text{i.e. Mean size} = \frac{l \times w \times RF}{N}$$

This value gives an indication of the individual histogram and can be used for direct comparison. By finding this value for every animal and then finding the average mean cell size for specific age groups a table (Table B, V.2) can be produced from which the graph (Fig.11, V.2) was plotted.

From the graph it can be seen that from before 30 days of age there is a highly marked difference in mean cell size, which is entirely dependant on strain as sex and age do not affect the readings at all. This is the first result which shows a difference between the two strains, where qualitatively none was visible to the microscopist. This difference in mean cell size is very useful in that it is independent of section thickness and is quite simple to produce.

However, analysis of this result raises more questions. A mean cell size difference can be due to a variation in cell size (and if so what sizes are involved) or a variation in cell number or both. Furthermore does this difference in mean cell size apply throughout the entire cortex, or is it produced by a localised change in number and/or size? The latter question can be answered by dividing the L_2 - L_6 by two and comparing the upper halves of the two strains, and then the lower halves of the two strains. If the change is localised then one half will be affected, whereas if the change is diffuse both upper and lower halves will show a difference when strains are compared.

The graphs (Figs. 12a, b, V.2) show a consistent difference after 30 days in mean cell size in both the upper and lower halves. This change again is dependent on strain, and not on sex or age.

A further interesting point is that the upper half of the cortex (i.e. $\frac{L_2 - L_6}{2}$) has a higher mean cell size than the lower half. This is surprising because from the lamination one would imagine this division to roughly fall between L_4 and L_5, and although L_5 contains the largest cells in the cortex, presumably the many small cells within L_6 bring down the overall mean cell size. The question posed by the mean cell size graph, namely, is the difference due to variation in cell size and/or cell number, can be answered by considering cell density.

(d) <u>Cell Density</u>

Cell density can be calculated from the total number of cells in a sample divided by the volume of that sample. This is a more complicated procedure than mean cell size as both the reduction factor and split cell correction factor enter into the calculation. However, in essence, the area which is quantitated can be found by multiplying the area of the microscope field or box by the total number of boxes required to traverse the cortex, i.e.

cortex depth. This is repeated 5 times so the total area counted is 5 x Area of box x cortex depth x RF as this is an area reduction.

To bring area to volume, however, the thickness of the section must be considered. The processing reduction which affects the area (R.F.) also reduces the thickness of the section and as this is a linear value, the $\sqrt{RF}$ or linear processing reduction factor must be used.

Thus Vol. counted = Area x thickness

= 5 x Area of box x No. of boxes ~~cortex depth~~ x RF x thickness of section x $\sqrt{RF}$ in μm^3.

The total number of cells in the known volume is given in the print outs, but this is too high due to split cells being counted as whole cells so the correction factor of Murphy discussed in Chapter II is used to find the corrected result.

Finally $\dfrac{\text{corrected No. of cells in sample}}{\text{corrected vol. of sample quantitated}}$ = No. of cells per unit vol.

This result can be calculated for each individual and is given in the print out (Figs. 9a, b, V.2) as "Corrected Density". This value again can be represented on a graph so that a comparison of the two strains can be made (Fig.13, V.2). The graph shows that the density decreased from birth to 30 days in both strains, and that both strains have similar densities during this period.However after 30 days the P.V.G. density continues to decrease and reaches adult level by roughly 50 days. The Campbell cortex density, however, behaves differently, apparently normal until 25 - 30 days, where it remains on a higher fluctuating level, never decreasing to the P.V.G. level.

The P.V.G. after 50 days remains remarkably constant. This would suggest that either the Campbell fails to develop from day 25 or that some extra cells make their appearance in the visual cortex of the blind animal, i.e.

$$\text{as density} = \frac{\text{corrected number of cells in sample}}{\text{corrected volume of sample quantitated}}$$

either the number or the volume must alter.

The volume quantitated is

5 x Area of box (field) x RF x No. of boxes to traverse cortex (i.e. cortex depth) x $\sqrt{RF}$. x t.

In this equation the only other variable which could alter due to the individual rat is the number of boxes required to traverse the cortex, i.e. the cortex depth.

(e) Cortex Depth

This value is obtained by use of the "Wild" drawing arm at a constant magnification as explained in Chapter III. The result obtained by this method is the "processed" depth of the visual cortex; by multiplying by the linear correction factor $\sqrt{RF}$ the corrected cortex depth can be found. These results are given in the print out (Figs.9a, b, V.2) as Thickness and Thickness x SQRT (R.F.) in the first row. The graph of these results is shown in Fig.14, V.2. Analysis of this graph shows that adult width or depth of cortex is attained in both species by 30 days of age and this value remains quite constant over the period of the graph. No difference in this dimension of the cortex is discernible between males and females or between blind and sighted rats. Thus the difference in density found when the Campbell and P.V.G. visual cortices are compared is not due to a difference in the volume of the cortex; it must be due to a difference in the number of cells per unit area.

The next step is to ascertain what sort of cells are involved in this density change, i.e. what size of cell? Table B on the print out provides a histogram of cell population for every individual. As explained before the number of histograms produced would make analysis difficult, but what can be compared are groups from each individual to see what sizes of cell are producing this change in cell density.

These values are all in arbitrary size units as no correction for π has been made at this stage. It was decided purely on a tentative basis to take 75 units as the testing size and to compare densities of the two strains > and <75 units. These results are produced on the print out at the bottom of Table B (Figs.9a,b, V.2).

(f) Density of cells of <75 and >75 units (Figs.15a, b, V.2).

From these two graphs it can be seen that the difference in overall cell density between the two strains appears to be due to small cells, i.e. cells of <75 arbitrary units. The P.V.G. results from day 30 remain remarkably constant in both graphs, and the Campbell cells of >75 units behave similarly. However, the cells of <75 units in both male and female Campbell visual cortices from day 30 fluctuate widely. Thus purely by taking this arbitrary level of 75 units as a test a difference in the small cells was shown. The next step was to attempt to use the data to find the separating units. As these results are in essence using histograms further subdivision is not possible. This helps to answer the original question posed after the mean cell size graph where the Campbell rat showed a smaller mean cell size, namely that variation in the number of small cells is responsible for this change. The other question posed after that graph concerned the large cells - if there were fewer large cells in the Campbell cortex this would also affect the mean cell size graph. It would seem from the graphs that the cells of >75 - 100 show very little strain difference in density.

(i) Density of Cells of 100 - <200 and 200 - 500 units(Figs.20a,b,V.2)

The graph of 100 - <200 units shows comparatively little difference between the strains. The 200 - 500 unit graph however, shows a strain difference, although with the reduction in cell numbers of these large cells the scale of this graph is much smaller than the previous graphs. The P.V.G. visual cortex nevertheless has a higher density of these large cells after 30 days. A further interesting point with this graph is that the overall density of these large cells tends to decrease slowly with age.

(j) Summary

In summary it would seem that there is a difference between the visual cortices of sighted and retinal dystrophic rats. This difference is not discernible qualitatively but is revealed by quantitation.

The steps in the quantitation were as follows - the mean cell size in the P.V.G. was higher than in the Campbell. This difference was found to be due to -

(α) An absolute increase in the number of smaller cells i.e. (<75 - 100) in the Campbell.

(β) Although there was very little difference in cell number of the large cells (i.e. >75 - 100) the largest cells (>200) are less frequent in the Campbell.

QUANTITATION Mean Cell Size	RESULT Difference after 30d.	CONCLUSION First difference found
" " " upper half	" " "	Difference between strains is diffuse
" " " lower half	" " "	
Cell Density	" " "	Part of difference in size is due to a difference in cell density.
" " <75	" " "	Absolute ↑ in cells of <75-100 units. Cells >75 units are very similar in density, but large cells of >200 units more common in P.V.G.
" " >75	No difference.	
" " G1-G6	Differences up to <100 units	
" " <100	Differences after 30 d.	
" " >100	Very little diff.	
" " >200	Difference after 30 d.	

SECTION 3. MISCELLANEOUS QUANTITATION

(a) Fixation

The importance of fixation throughout this work has been emphasised, and before the standard perfusion method had been fixed an experiment was carried out to measure the effect of different fixatives on the volume of a brain block. The volume of the block was found by weighing the brain in air and then in water and using the formula

$$\text{Vol} = \frac{\text{Wt. in air} - \text{Wt. in water}}{\text{Specific gravity of water}}$$

The effect of the following fixatives was found (Culling, 1963).

(i) 10% unbuffered formalin

(ii) 10% neutral buffered formalin - 6 months old

(iii) 10% neutral buffered formalin - freshly made

(iv) Bouins fixative

and volumes were measured daily for 3 weeks.

The results were plotted on a graph (Fig.21, V.2) which shows the large variation in final volumes. The effect of the ageing of buffered formalin stresses that fresh solutions must be used.

The conclusion derived from the graph is that for quantitation, a standard fixation technique must be used. The graph also raises the problem of comparing results obtained with one fixative against those in the literature where a wide variety of fixatives have been used.

(b) Reduction Factor

The importance of the reduction factor in dealing with processing variations has also been mentioned. A graph was drawn of the R.F. against age, with sex and strain as variables. Again the wide variation in the reduction factor is shown (Fig.21, V.2) but no conclusion can be drawn of any correlation with age, strain or sex, because of the many variables discussed in Chapter II.

(c) Experimental Rats Treated with Retinotoxic Drugs

Representative counts of the visual cortices of these animals both P.V.G. and Campbell failed to show any divergence from the results of the untreated animals. As no pathological changes were seen in the eyes either, it can be presumed that at the dose level and the route of administration used these drugs are not retinotoxic in these rats.

SECTION 4. STATISTICS

(a) Introduction and Method

The aim of the statistics was firstly to determine the significance, if any, of the differences found between the strains, and secondly, to verify that a change can be demonstrated after 30 days of age.

The quantitation was performed on an age basis, i.e. for each feature measured at each age there are results for each strain and sex, i.e. for C♂ C♀ P♂ and P♀. These results can be ranked for each age e.g. for cortex depth in 30 day old ♂

P♂ 1316 µm (+ve)

C♂ 1448 µm

the C♂ has the greater value, and so receives a +ve. This ranking is then repeated throughout the range of ages quantitated. In the total counted, if the cortex thickness was the same for both strains, the chance of a +ve being given is equal for both strains. In other words, the probability that for a particular pair the +ve is given to the C strains equals $\frac{1}{2}$.

Using cortex thickness in a total of 43 males the results obtained were P♂ 22 C♂ 21. However, it was decided to see if the 30 day change could be verified statistically so results were divided into <30 days and >30 days.

Ranking cortex thickness results for <30 and >30 days.

Group	Total	Observed Ratio P:C
♂ <30 days	23	11:12
♂ >30 days	20	11:9

To evaluate the significance the binominal distribution confidence limits are used (Geigy Scientific Tables p.106). In a total of 23 pairs of values a "split" of 6-17 (or 17-6), or 18-5 can be considered significant at the 5% level and a split of 4-19 (or 19-4, and more extreme 20-3, 21-2, 22-1, 23-0) would be significant at the 1% level. These results, therefore, show that there is no difference in visual cortex thickness between the two strains, i.e. it is not significant.

However, if density of cells is examined, again in males the following results are obtained.

Group	Total	Observed Ratio P:C
♂ <30 days	23	10:13
♂ >30 days	20	1:19

The density at <30 days is not significant but, at >30 days in a total of 20, the 1% significance level is <u>3-17</u>.

So the result of 1:19 for density of cells in >30 day rats is highly significant.

(b) Summary of Results and Significance

N.S. = Not significant.

1. Mean Cell Size

(i) Total cortex

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	14:9	6-17	N.S.
♀ <30 days	14:9	6-17	N.S.
♂ >30 days	20:0	5-15	Significant at 1% level
♀ >30 days	20:0	5-15	Significant at 1% level.

(ii) Upper half cortex

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	15:8	6-17	N.S.
♀ <30 days	10:13	6-17	N.S.
♂ >30 days	20:0	5-15	Significant at 1% level
♀ >30 days	19:1	5-15	Significant at 1% level.

(iii) Lower half cortex

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	14:9	6-17	N.S.
♀ <30 days	13½:9½	6-17	N.S.
♂ >30 days	20:0	5-15	Significant at 1% level
♀ >30 days	19½:½	5-15	Significant at 1% level.

2. Cortex Thickness

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	11:12	6-17	N.S.
♀ <30 days	7:16	6-17	N.S.
♂ >30 days	11:9	5-15	N.S.
♀ >30 days	11:9	5-15	N.S.

3. Density of Cells

(i) No. of cells/mm^3

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	10:13	6-17	N.S.
♀ <30 days	13:10	6-17	N.S.
♂ >30 days	1:19	5-15	Significant at 1% level
♀ >30 days	3:17	5-15	Significant at 1% level.

(ii) No. of cells (<75 units) /mm^3

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	9:14	6-17	N.S.
♀ <30 days	8:15	6-17	N.S.
♂ >30 days	0:20	5-15	Significant at 1% level
♀ >30 days	1:19	5-15	Significant at 1% level.

(iii) No. of cells (>75 units) /mm^3

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	13:10	6-17	N.S.
♀ <30 days	16:7	6-17	N.S.
♂ >30 days	10:10	5-15	N.S.
♀ >30 days	8½:11½	5-15	N.S.

(iv) No. of cells (<100 units) /mm^3

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	9:14	6-17	N.S.
♀ <30 days	10:13	6-17	N.S.
♂ >30 days	0:20	5-15	Significant at 1% level
♀ >30 days	1:19	5-15	Significant at 1% level.

(v) No. of cells (>100 units) /mm^3

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	12:11	6-17	N.S.
♀ <30 days	13:10	6-17	N.S.
♂ >30 days	15:5	5-15	Significant at 5% level
♀ >30 days	17:3	5-15	Significant at 1% level

(vi) No. of cells (100 - 200 units) /mm^3

♂ <30 days	12:11	6-17	N.S.
♀ <30 days	16:7	6-17	N.S.
♂ >30 days	16:4	5-15	Significant at 5% level
♀ >30 days	14:6	5-15	N.S.

(vii) No. of cells (>200 units) /mm^3

♂ <30 days	14:7	5-16	N.S.
♀ <30 days	12:9	5-16	N.S.
♂ >30 days	14½:5½	5-15	N.S.
♀ >30 days	19:1	5-15	Significant at 1% level.

4. <u>Group Density</u>

(i) G1 (<25 units) cells/mm^3

♂ <30 days	8:15	6-17	N.S.
♀ <30 days	7:16	6-17	N.S.
♂ >30 days	1:19	5-15	Significant at 1% level
♀ >30 days	4:16	5-15	Significant at 5% level.

(ii) G2 (25 - <50 units) cells/mm^3

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	10:13	6-17	N.S.
♀ <30 days	10:13	6-17	N.S.
♂ >30 days	2½:17½	5-15	Significant at 1% level
♀ >30 days	3:17	5-15	Significant at 1% level.

(iii) G3 (50 - <70 units) cells/mm^3

♂ <30 days	11:12	6-17	N.S.
♀ <30 days	13:10	6-17	N.S.
♂ >30 days	3:17	5-15	Significant at 1% level
♀ >30 days	3:17	5-15	Significant at 1% level.

(iv) G4 (75 - <100 units) cells/mm^3

♂ <30 days	10:13	6-17	N.S.
♀ <30 days	12:11	6-17	N.S.
♂ >30 days	3:17	5-15	Significant at 1% level
♀ >30 days	0:20	5-15	Significant at 1% level.

(v) G5 (100 - <125 units) cells/mm^3

♂ <30 days	10:13	6-17	N.S.
♀ <30 days	12:11	6-17	N.S.
♂ >30 days	5:15	5-15	Significant at 5% level
♀ >30 days	4:16	5-15	Significant at 5% level.

(vi) G6 (125 - <150 units) cells/mm^3

Group	Observed Ratio P:C	5% Significance	Conclusion
♂ <30 days	11½:11½	6-17	N.S.
♀ <30 days	12:11	6-17	N.S.
♂ >30 days	14:6	5-15	N.S.
♀ >30 days	16:4	5-15	Significant at 5% level.

The statistical findings thus compliment the results which have been presented graphically. None of the variables showed any significant difference before 30 days, and those variables which showed a clear strain difference after 30 days on the graphs were found to be significant at the 1 - 5% level. It is surprising that in the category "No. of cells of >200 units/mm^3" that the >30 days C♀ result should be significantly different at the 1% level, whereas the >30 days C♂ result is not significantly different and yet the graph of these results (Fig.20b,V.2) shows clear differences. The result for the males P♂: C♂ was 14½:5½, which although it does not quite achieve the 5% significance level (15:5), is a good indication of a similar change to that found in the females for this variable. The smaller sample used for this result as the scale of the graph (Fig. 20b,V.2) shows, may play a part in this unexpected result, although it may be that the difference between the strains in the case of large cells in the males is not as great as the difference using other variables.

SECTION 5. SUMMARY

In summary the main differences between the visual cortices of blind and retinal dystrophic rats, found by quantitation are :

(A) An increase in the number of small cells ($<79\mu m^2$) in the blind visual cortex. These cells could be either glia, endothelial cells or small neurones - the quantitation performed does not distinguish what type is involved. This difference is significant at the 1% level.

(B) A decrease in the number of large cells ($>147\mu m^2$) in the blind visual cortex. This decrease is quite small compared to the increase in small cells, and it would seem as if full development had not occurred. These large cells are probably neurones. This difference is significant at the 1% level in females, but not in males.

These changes are seen after 30 days; before this age no difference can be seen when Campbell and P.V.G. visual cortices are compared. No effect whatsoever on the quantitation results was produced by the drugs used.

CHAPTER VI

IDENTIFICATION OF THE CELLS INVOLVED IN THE CHANGES IN THE CORTEX

		Page
SECTION 1.	QUANTITATIVE APPROACH	
	(a) Transformation of Arbitrary Units to Absolute Units	155
	(b) The Problem of Cell Number Changes in Relation to Area Observed....	156
SECTION 2.	QUALITATIVE ELECTRON MICROSCOPE INVESTIGATION	
	(a) Introduction	158
	(b) General	158
	(c) Neurones	159
	(d) Astrocytes and Oligodendroglia ...	160
	(e) Microglia	160
	(f) The Origin and Function of Microglia	162
	(g) Summary	163
SECTION 3.	QUALITATIVE LIGHT MICROSCOPE INVESTIGATION	
	(a) Introduction	165
	(b) Cell Identification	167
	(c) Cell Inclusion Identification	168
SECTION 4.	SUMMARY	170

CHAPTER VI

SECTION 1. QUANTITATIVE APPROACH

(a) Transformation of arbitrary units to absolute units

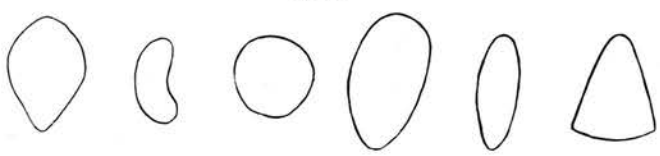
(b) The problem of cell number changes in relation to area observed

(a) Transformation of Arbitrary Units to Absolute Units

The first step in identification of the cells involved in the visual cortex changes was to try and get the measurements into absolute units as against arbitrary units as used in Chapter V. The size of the cells was taken as l x w x RF, as a wide range of cell shapes were measured.

Diag. A

CORTEX CELL SHAPES



Counted Area	A	131	58	102	175	74	120
l×w Area	l×w	188	62·5	132	220	102	180
Factor = $\frac{A}{l \times w}$	$\frac{\pi}{4}$ = 0·775	0·700	0·930	0·770	0·795	0·730	0·660

The nearest approximation of all the shapes is a spheroid, which has the formula for cross sectional area A , $A = \pi \frac{l}{2} \times \frac{w}{2}$. Correcting this for processing the formula now is $A = \frac{\pi}{4} (l \times w \times RF)$. Thus the arbitrary units used in quantitation should be multiplied by a factor $\frac{\pi}{4}$ to bring the results to μm^2. This is obviously an approximation, particularly with the unusually shaped cells but it helps to give an idea of the cell size involved. The cell size ranges involved in the changes after 30 days are:

(a) 0 - 100 A. Units = 0 - 79 μm^2

(b) > 200 " " = > 147 μm^2

The frequency of these cells is also known (over 30 days of age) in R.F. corrected tissue.

			Campbell
0 - 79 μm^2	P.V.G.	= 425×10^2 cells / mm^3	= 95% ↑
	Campbell	= 832×10^2 " "	
> 147 μm^2	P.V.G.	= 59×10^2 " "	= 60% ↓
	Campbell	= 20×10^2 " "	
All Cells	P.V.G.	= 897×10^2 " "	= 32% ↑
	Campbell	= 1188×10^2 " "	

(b) <u>The Problem of Cell Number Changes in Relation to Area Observed</u>

These changes seem quite high, but the microscopist is viewing the section in which all the cells are present, and the alteration is difficult to see as the increase in small cells will be somewhat cancelled by the decrease in large cells. Furthermore, the field visible on the microscope decreases in area as magnification increases.

Eyepiece	Obj.	Radius of field	Area of field	Volume	If 10M F/S
x12.5	x20	0.45 mm	$.656mm^2$	$.0040\ mm^3$	$.00656\ mm^3$
x12.5	x40	0.215 mm	$.145mm^2$	$.00085\ mm^3$	$.00145\ mm^3$
x12.5	x100	0.09 mm	$.0252mm^2$	$.000155\ mm^3$	$.000252\ mm^3$

If thickness section 5μ
with RF of 1.5
thick = $5 \times \sqrt{1.5} = 6.1\mu$.

Using 10μ frozen sections to avoid R.F. complications.

Obj.	Vol.tissue/field	If 900×10^2/cells $/mm^3$ Then cells/field	If 5μ F/S cells/field
x20 obj	$.00656\ mm^3$	585	292
x40 obj	$.00145\ mm^3$	135	62
x100 oil	$.000252\ mm^3$	22	11

Using these high magnifications necessary to see cell morphology, it is difficult to see an increase in the cell population of the range of 32%, particularly in a tissue like the rat cortex which has varying degrees of cellularity, and where the 32% increase in cell density is not localised, but is spread throughout the cortex.

To identify the cells involved in the changes it was decided to use two methods - namely electron microscopy and light microscopy, in the latter utilizing special stains on frozen and paraffin sections.

SECTION 2. QUALITATIVE ELECTRON MICROSCOPE INVESTIGATION

(a) Introduction

(b) General

(c) Neurones

(d) Astrocytes and Oligodendroglia

(e) Microglia

(f) The origins and function of microglia

(g) Summary

(a) Introduction

It seems a contradiction to use the ultra thin sections of electron microscopy to search for an increase in cells which cannot be recognised in paraffin section. This, however, was not the immediate aim; the purpose in fact was to examine and photograph the cells concerned (i.e. $<79\mu m^2$ and $>147\ \mu m^2$), in both the normal and blind cortices to identify the cells and see if any fine structure differences were visible. In addition the remainder of the cortex i.e. the neurophil could be examined for condition of myelin, and the appearance of the blood vessels.

Electron microscope sections and 1μ thick araldite sections for light microscopy were prepared as explained in Chapter III.

Sections from 30, 70, 300 and 500 days old P.V.G. and Campbell visual cortices were examined.

(b) General

Electron microscopy of the rat visual cortex reveals a neurophil of closely packed axons, dendrites and glial processes with the associated neurones, glia and endothelial cells. These fractions can be identified, albeit with some difficulty where the descriptive criteria tend to be ambiguous.

(c) Neurones

Neurones in the visual cortices of Campbell and P.V.G. rats show three distinct divisions - the cell body, dendrites and the axon (Figs. 23a,b; 24a,b,V.2). Most of the cell body or perikaryon is occupied by the large rounded nucleus composed of relatively homogeneous karyoplasm and, depending on the plane of section, a nucleolus (Fig.23a, V.2). The rest of the perikaryon, the cytoplasm, contains the granular and agranular endoplasmic reticulum with the granular nodal points, or Nissl bodies and the agranular configuration, the Golgi apparatus. Ribosomes, mitochondria, microtubules, multivesicular bodies, lysosomes and lipofuscin granules can also be recognised. Stellate and pyramidal neurones can be distinguished by their perikaryon shapes, and the nuclear outline which shows clefts in stellate cells (Fig.23a,b, V.2).

From the quantitation results it would appear that there is little or no difference in density of cells of $>79\mu m^2$ but there is a 60% decrease in density of the largest cells of $>147\mu m^2$ in the Campbell. These large cells must be neurones so nerve cells of known location (i.e. in lamina if visible, or as fraction of total cortex e.g. ½way through cortex) were compared at different ages. No qualitative differences could be seen. A quantitative examination of these electron micrographs was not made, as this would have required a special quantitative electron micrograph technique, considering the small sample that could be measured, and the fact that the cell organelles which would be quantitated appear to vary in their distribution throughout the cytoplasm, e.g. the reduction in Nissl substance at the axon hillock. No degenerating neurones were visible in any of the sections.

(d) Astrocytes and Oligodendroglia

Protoplasmic astrocytes and oligodendroglia were recognised in both strains, again with no obvious fine structure difference. Astrocytes were identified by their relatively electron translucent appearance, bundles of cytoplasmic fibrils, sparse endoplasmic reticulum, glycogen granules and irregular outline. Astrocytes were uncommon in both visual cortices. Oligodendroglia vary in electron density, depending on type, but have an irregularly shaped nucleus showing some clumping of the nuclear chromatin. The endoplasmic reticulum is well developed; many ribosomes are found free in cytoplasm and lining the nuclear membrane; in addition numerous microtubules can be seen (Peters et al, 1970; Matthews and Kruger, 1973b). Again no difference in fine structure or density of astrocytes and oligodendroglia could be seen, when those from the sighted and blind cortices were compared.

(e) Microglia

The other neuroglial cell present in the cortex is the microglia - which in light microscopy is small, darkly staining with a scanty cytoplasm. These cells from light microscope preparations are said to comprise 10% of the neuroglia cells of the cortex (Peters et al, 1970). The criteria for identification of this cell are uncertain, earliest reports having arrived at neuroglia by eliminating other cells (Schultz et al, 1957). Other workers have tended to classify microglia as a type of oligodendroglia (Kruger and Maxwell, 1966), or as spongioblasts (Caley and Maxwell, 1968b). However the generally accepted criteria for electron micrograph identification are a somewhat flattened nucleus with clumped chromatin, very few microtubules (distinct from oligo) and no filaments or glycogen (distinct from astrocytes). The granular endoplasmic reticulum consists of long strands which wind through the cytoplasm. Dense laminar bodies and homogenous droplets probably lipid are often present in the cytoplasm. The cell outline is not very clear (Peters et al, 1970) (Figs.25a,b; 26a,b; 27a,b, V.2).

The most striking feature of the Campbell retinal dystrophic cortex was that these cells although they must be present could not be identified in the 30 day old animal but could be found quite easily in the 300 and 500 day olds, and with some difficulty in the 70 day old. In the P.V.G. the earliest this cell was seen was 300 days and only after an extensive search. Microglia were again difficult to find in the 500 day old P.V.G. visual cortex.

Thus the arbitrary qualitative assessment that microglia were much easier to find in the older blind rats, suggests these cells have a higher density in these animals; and in fact a much higher density than in the P.V.G. as this difference can be seen in the very small samples of tissue used in electron microscopy.

These cells in both strains contained dense bodies and lipid vacuoles. The 70 day old Campbell microglia tend to contain more dark lysosomal material (Fig. 27a, V.2) than the older rat microglia which contain more lipid (Fig. 25b, V.2). Microglia can be found in the normal 300 day old P.V.G. cortex (Fig. 26a, V.2) and these cells again contain dense bodies and lipid inclusions, but not to the same extent as in the Campbell. These cells were not observed engulfing material, but show phagocytosed debris which is in a state of lysis (Fig. 27b, V.2). There is a lamination visible in some of this degenerating material, which resembles myelin superficially.

(f) The Origin and Function of Microglia

This finding raised two problems which suggested further electron microscopy - namely where did these cells come from, and what is the source of the debris in these cells?

The source of these cells has been the subject of controversy for some time, but in summary they either form from existing quiescent microglia cells, from multipotential glia (Vaughn and Peters, 1968) or from perivascular and haematogenous cells (Matthews and Kruger, 1973a, b; Konigsmark and Sidman, 1963; Maxwell and Kruger, 1965). It would appear that during the resting stage these cells are difficult to distinguish from immature ectodermal derivatives such as multipotential glia or even oligodendroglia.

However, by 70 days of age in the Campbell visual cortex these cells appear to have proliferated and to have phagocytosed debris (Fig. 27a, V.2) - therefore if these cells develop from existing cells some form of cell division should be seen, or if from vascular elements some change should occur around blood vessels.

No division of any existing cellular elements was seen, and although pericytes seemed prominent in the Campbell visual cortex (Fig.28a,b, V.2) the variety of forms was difficult to classify, and these types could also be seen in the P.V.G. Lipid inclusions and dense bodies could be seen in the cytoplasm of these perivascular cells again in both species (Fig. 28b, V.2). The wide variety of these pericyte shapes and sizes, and the variation in amount of inclusions within the one specimen made any assessment very difficult. No migration of haematogenous cells through vessels was seen in either strain, and the origin of these cells must remain unknown.

The source of the debris remains unclear - no degenerating neurones were found, and the microglia do not appear to have any specific location - they were found as neurone satellites, free in the tissue and perivascularly. Myelin figures of bizarre forms were found occasionally in the Campbell cortex (Fig. 29, V.2) and not in the P.V.G. but not enough for a quantitative assessment.

(g) Summary

Thus in summary, no qualitative differences in large cells, i.e. neurones, could be found on electron microscopy, when the normal and retinal dystrophic visual cortices were compared.

However, microglia were found to be more frequent in the Campbell visual cortex from 70 days, and it would seem some sort of change has stimulated this microglial response, and nothing else, no astrocytes or oligodendroglia were involved. No degenerating neurones were found, and except for some myelin ovoids (Fig.29, V.2) no evidence of any change which might stimulate microglia to proliferate and phagocytose was uncovered.

The value of the electron microscopy was that through it tentative identification of the cells involved in the change was possible, namely microglia and possibly vascular and perivascular elements. Furthermore the microglia have phagocytosed lipid material and this may stain with fat stains on frozen section. The lack of astrocyte and oligodendroglia involvement in these changes can also be confirmed by special stains.

SECTION 3. QUALITATIVE LIGHT MICROSCOPE INVESTIGATION

(a) Introduction

(b) Cell identification

(c) Cell inclusion identification

(a) Introduction

From the results obtained by quantitation and electron microscopy it would seem that in the Campbell visual cortex there are two factors contributing to the differences from the P.V.G. The first is an apparent lack of full development of the large neurones, and the second a small cell reaction, which according to the electron micrographs is microglia and possibly pericytes.

Investigation of these changes involved performing special stains on paraffin and frozen sections, for neurones and for glial cells. The special stains for glia were found highly capricious, and even with absolute cleanliness of glassware, and scrupulous adherence to the suggested times, unsatisfactory results were often obtained.

The following stains were used :

	STAIN	RESULT	REFERENCE
General	Haematoxylin and Eosin		Drury and Wallington 1967
	Goldners Trichrome		Disbrey and Rack 1970
	Acid phosphatase		Bancroft, 1967
Neurones	Toluidine Blue	Nissl and nuclei - blue	Disbrey and Rack, 1970
	Cresyl fast Violet	" " " - violet	" " " 1970
	Gallocyanin		Drury and Wallington 1967
Myelin	Luxol fast Blue	Myelin sheaths - blue	Disbrey and Rack, 1970
Degenerate Myelin	Osmium tetroxide and napthalamine (O.T.A.N.)	)Deg.myelin)- black)	Drury and Wallington, 1967
	Marchi	Deg.myelin - black	Culling, 1963
Astrocytes	Cajal Gold chloride sublimate	Astrocytes - purple black	Drury and Wallington, 1967
	Hortega silver carbonate	Astrocytes - brown black	" " "
	Phosphotungstic Acid haematoxylin	Astrocytes, nuclei - blue	" " "
	Victoria Blue	Fibrous glia, nuclei - blue	" " "
Oligodendroglia	Hortega silver carbonate	Oligodendroglia - black	Drury and Wallington, 1967
	Penfield's combined oligodendroglia and microglia method	Oligodendroglia and microglia - black	" " "

contd ...

Microglia	Hortega silver carbonate	Microglia - black	Drury and Wallington, 1967
	Periodic acid Schiff (P.A.S.)	P.A.S. +ve material - bright red	Culling, 1963
	Nile Blue Sulphate	Acidic lipid - blue	Bancroft, 1967
		Non acidic lipid - red	" "
	Performic Acid Schiff	Unsat. lipid - red	" "
	Sudan Black	Lipid - black	" "
	Scharlach R.	Lipid - red	Gasser, 1961

(b) Cell Identification

The special stains for neurones failed to reveal any difference between the neurones of both strains. The acid phosphatase result was inconclusive.

The special stains for astrocytes and oligodendroglia revealed no difference on a qualitative basis between the P.V.G. and Campbell, and no gliosis was found in either strain with P.T.A.H. or Victoria Blue. Hortega's method for microglia was found to stain some oligodendroglia as well, and despite many attempts invariably unsatisfactory, preparations for quantitation were produced. Microglia and oligodendroglia could be identified, but there were always areas where identification was impossible due to non specific staining. Furthermore no microglia could be seen which had inclusions, i.e. when these cells increase in size and become gitter cells, it would appear they fail to stain with silver.

This unsatisfactory result prompted another route - namely staining the inclusions, and so indicating the cell. The stain for normal myelin revealed no difference between the strains, but electron microscopy had shown some unusual myelin figures in the blind visual cortex, so stains for degenerating myelin were tried, but failed to show any degenerate myelin.

(c) Cell Inclusion Identification

The O.T.A.N. stain for degenerate myelin failed to stain any degenerating myelin, but did however stain the microglia (when the fixative was altered from neutral buffered formalin to Baker's formalin). The microglia nucleus stained blue with the counter-stain, but the cytoplasmic inclusions stained a golden black against the background of red-black. As 15μm sections were used, a lot of fine focusing under oil immersion was needed to locate these cells in the Campbell visual cortex. These cells could also be located in the P.V.G. cortex, but as they were present in much smaller numbers, they were extremely difficult to find.

Further fat stains were performed - oil red O, Sudan black, Scharlach R, which again with varying degrees of difficulty, confirmed the presence of the lipid inclusions in these microglia. Identification of these lipid inclusions was tried, using the routine in Bancroft (1967) but the results were ambiguous. The problem was identifying a lipid inclusion in a section which contains many other lipids, which are much more prominent than the unknown lipid. However, the lipid inclusions were found to stain green with Nile Blue sulphate - which suggests a mixture of acidic (blue) and non acidic (red) lipids. These inclusions were also Periodic Acid Schiff (P.A.S.) positive (Figs.30a, b, V.2) which meant they could be recognised in paraffin as well as frozen sections. Myelin is a complex of lipids (Elliot, Page and Quartel, 1962) and in degeneration of myelin the cerebroside (Disbrey and Rack, 1970) and carbohydrate content of myelin react with P.A.S. P.A.S. also reacts with the basement membrane of blood vessels, and the lipofuchsin in neurones, but nevertheless this stain proved very useful in confirming the higher incidence of microglia with inclusions in the Campbell cortex. P.A.S. positive material in the form of vacuoles was present in the cytoplasm of the endothelial and perivascular cells, but no conclusion could be drawn as to degree because of the large amount of variation in both cortices. A P.A.S. +ve tract was found in the cortex of a blind rat (Fig. 30b, V.2) and this tract may represent the path of migration of a microglia to a blood vessel.

SECTION 4. SUMMARY

In summary, the study of the light microscopic special stains and the electron micrographs confirmed the quantitative result of an increase in the number of small cells in the retinal dystrophic cortex but failed to show any difference in the large cells. The small cells were identified as microglia with a possible perivascular involvement. Even in specially stained sections these cells were difficult to see and without the pointers from quantitation, this difference would easily have been overlooked. The conclusion drawn from these results is that there is an alteration in the blind visual cortex which may be due to a failure of full development of neurones in the Campbell cortex, and which stimulates only a microglial response, with possible perivascular involvement.

CHAPTER VII

MISCELLANEOUS PATHOLOGY

		Page
SECTION 1.	INTRODUCTION	172
SECTION 2.	PATHOLOGY OF RETINOTOXIC DRUGS	173
SECTION 3.	BACKGROUND PATHOLOGY	
	(a) Chronic Respiratory Disease	176
	(b) Otitis Media	177
	(c) Sialodacryoadenitis	178
	(d) Hydronephrosis	178
	(e) Glomerulonephritis	180
	(f) Cysticercus Fasciolaris	180
	(g) Anophthalmia	180
	(h) Peromelia	181
	(i) Conclusion	181

CHAPTER VII

SECTION 1. INTRODUCTION

The quantitation carried out in this work necessitates the use of as "standard" animals as possible and the significance of other diseases complicating the outcome must be considered. A postmortem examination was performed on every animal perfused, and a further histological investigation on selected specimens. Histological examination was also carried out on the experimental animals used to test the retinotoxicity of the three drugs used in this work.

SECTION 2. PATHOLOGY OF RETINOTOXIC DRUGS

The three drugs listed below were used in this work at the dose rates given.

		Dose Rates	
968A	1, 5 - Di - (p- aminophenoxy) pentane dihydrochloride hydrate	10 mgm	20 mgm/Kg
2948A	1 - p - Aminophenoxy - 5 - phthalimido-pentane	10 mgm	20 mgm/Kg
Na I	Sodium Iodoacetate	60 mgm	20 mgm/Kg

These drugs were injected intraperitoneally into 1 day old and adult rats to test for retinotoxicity. At the dose rates shown, these drugs failed to cause any ophthalmoscopic signs of retinotoxicity and histological examination of the eyes and central nervous system was also negative. The eyes and C.N.S. of these animals were fixed by perfusion and because of the artefacts caused by the artery forceps closing the posterior vena cava and posterior aorta, some difficulty was found in interpreting the sections from abdominal organs. These artefacts, due to perfusion present and venous congestion, were much more extensive in young animals, probably because the connective tissue fibres are not yet mature. The rat at 3 days shows signs of immaturity not only in the retina and C.N.S. (Chapters IV and V), but also the lungs, kidneys, pancreas, liver and spleen - the lungs are not fully expanded, the kidneys exhibit a nephrogenic cap and the pancreas is composed mainly of endocrine gland, whereas the exocrine portion is small and poorly developed. The glycogen rich liver exhibits extramedullary haematopoiesis, as does the spleen. No lymphocytes can be seen in the spleen at this age.

Chronic respiratory disease and hydronephrosis were seen in both control and experimental rats, and will be considered later. The individual toxicity of the drugs was as follows:

<u>968A</u> <u>10 mgm/Kg</u> - one 3 day old experimental rat showed swollen villi tips, with irregular eosinophilic vacuoles, although the brush border was intact.

<u>20 mgm/Kg</u> - No abnormalities detected.

<u>2948A</u> <u>10 mgm/Kg</u> - This drug was made up in an acid solution and both experimental and control rats showed a low grade peritonitis, shown by a slight thickening of the liver capsule and occasional polymorphonuclear leucocytes in the mesentery. Some fat necrosis with fibrosis could also be found. The only other finding was a centrilobular fatty change in the liver of a control rat.

<u>60 mgm/Kg</u> - At this dose rate a low grade peritonitis was found in both control and experimental rats, similar to that seen at the lower dose rate. The fact that the peritonitis was low grade in both experiments, and as such independent of dose rate, would suggest that the acid solvent used for this drug was responsible for the pathological findings and the drug had little or no effect.

<u>Na I</u> <u>60 mgm/Kg</u> - This high dose rate was found to be irritant, causing a more severe peritonitis than seen with 2948A, as

shown by the polymorphonuclear leucocytes and macrophages found along the mesenteric surface of most of the abdominal organs. A fringe of necrotic liver cells and inflammatory cells lined the liver, but had not penetrated the parenchyma. Small colonies of coccibacilli were found scattered throughout the tissue and it would appear that the freshly distilled water used as a solvent for all the drugs was not sterile, and that this high dose rate in this case lowered the resistance of the rat sufficiently to allow the bacteria to become established. No bacteria were found in any of the controls or with any of the other drugs.

10 mgm/Kg - No abnormalities detected.

These drugs, when given intraperitoneally, with the exception of Na I at the high dose rate of 60 mgm/Kg, were relatively non toxic in the rat at the dose rates shown. The acid solvent used to produce the solution of the second drug 2948A, caused a low grade peritonitis and as there was no difference between test and controls it would seem the drug itself made little or no difference.

The lack of retinotoxicity in the rat is probably due to the inherent resistance of the rat eye to damage by drugs although the drugs, dosage rates, method of administration and environmental factors will also contribute to this result.

SECTION 3. BACKGROUND PATHOLOGY

(a) Chronic Respiratory Disease (C.R.D.)

This can be defined as "a single disease which encompasses many lesions and syndromes including infections, catarrh and chronic murine pneumonia" (Lindsey et al, 1971). These authors contend that *Mycoplasma pulmonis* is the primary pathogen.

The clinical signs of C.R.D. in the rat are snuffling, with red encrustations around the nares and eyes, due to nasal and Harderian gland involvement. A humped posture and roughed coat are also seen. On postmortem examination the affected areas of the lung are rubbery, shrunken, red-grey-brown in colour with occasional multiple yellow foci. Microscopically the lung has an increase in the peribronchial and peribronchiolar lymphoid cells (Figs.30a, b, c, V.2). Increased mucus formation and metaplasia of the respiratory epithelium to a multinucleated layer, 6-7 cells thick was found, but no squamous epithelium was ever seen. Bronchiectasis with ulceration, and replacement by granulation tissue leading eventually to abscess formation (Figs.24a, b, V.2) could be seen occasionally.

The incidence of C.R.D. was compared in test and control animals and found to be

C.R.D.	Control	5/33	= 15%
<30 days	Test	9/35	= 26%
C.R.D.	Control	24/27	= 90%
>30 days	Test	35/39	= 90%

To test the significance of these results the "two-tailed test of significance" (Walker and Lev, 1953) was used. This has the formula.

$$z = \frac{N_2 n_1 - N_1 n_2}{\sqrt{\frac{N_1 N_2 n\,(N-n)}{N}}}$$

n_1 = no +ve in 1st sample i.e. control = 5

n_2 = no +ve in 2nd sample i.e. test = 9

N_1 = Total in 1st sample i.e. control = 33

N_2 = " " 2nd sample i.e. test = 35

$n = n_1 + n_2 = 10$

$N = N_1 + N_2 = 68$

At a significance level of 0.05 the region of acceptance is $-1.645 < z < 1.645$. The value for z using the above test on the C.R.D. results is 1.07, i.e. these results are not significantly different.

The incidence of C.R.D. in both strains of rats was found to be about 90% after 30 days of age. Abscess formation (Figs.32a, b, V.2) was only seen in two animals, both over 400 days. No strain or sex difference was found.

(b) <u>Otitis media</u>

Otitis media or middle ear disease (M.E.) can also be included in the Chronic Respiratory Disease complex (Lindsey et al, 1971). The significance of M.E. is that it can spread and involve the meninges and C.N.S. and complicate the quantitative results.

A careful search was performed in every C.N.S. dissection for the characteristic sign of M.E. namely purulent exudate in the tympanic cavity, and if any was found the specimen was discarded. In the animals in which no macroscopic evidence of M.E. was detected, i.e. the rats used for quantitation (Chapter V)., histological examination failed to reveal any involvement of the meninges or C.N.S.

Any animal which showed clinical signs of M.E. (tilting of the head) was culled from the colony.

(c) Sialodacryoadenitis

Sialodacryoadenitis or inflammation of the salivary and Harderian glands characterised by intermandibular oedema and red staining of the eyelids due to excessive porphyrin excretion has been described (Innes and Stanton, 1961; Hunt, 1963; Jonas et al, 1969). Although some red encrustation could be seen around the eyes of the blind animals no inflammation of the Harderian gland was ever found, histologically, and no involvement of the eye or infraorbital lacrimal gland was seen. The red stains around the eye could be due to porphyrins without any associated inflammation of the gland. This condition is often associated with C.R.D.; (Lindsey et al, 1971).

(d) Hydronephrosis

Unilateral or bilateral hydronephrosis was found in about 10% of the rats of both strains under 30 days, i.e. 3% of all ages (15/400). The figures found in the experimental and control rats were

Hydronephrosis	Control	3/33	= 9%
<30 days	Test	7/35	= 20%

and using the "two tailed test of significance" as for C.R.D. a result for z of 1.3 was found i.e. the difference is not significant.

In the animals of <30 days, there appeared to be a higher incidence in males (10 in a total of 15), although using the"one tailed test of significance" this was not significant at the 0.05 level where

$$z = \frac{p - P}{\sqrt{\frac{P \times Q}{N}}}$$

where p = Proportion found = .66

P = Proportion expected if no difference = .5

Q = $(1 - P)$

N = No of observations in total samples

(Walker and Lev, 1953).

$$z = \frac{.66 - .5}{\sqrt{\frac{.5 \times 5}{15}}} = 1.33$$

To be significant z should be greater than 1.645 at the .05 level. The result of 1.33 is not significant.

This 10% incidence in animals of <30 days, and not at all in the older rats would seem to suggest that the condition might regress. No obvious obstruction could be found along the ureter or urethra, and although various causes such as prostatitis or compression between the ileolumbar and spermatic or ovarian vessels have been advanced (Snell, 1967) no conclusions could be drawn.

(e) Glomerulonephritis

Two cases were recorded both in Campbell rats, of >400 days. Dilated distal convoluted tubules in the cortex and collecting tubules in the outer medulla (Fig.32c, V.2); both contained eosinophilic proteinaceous material. Polymorphonuclear leucocytes could be found in the glomeruli. No vascular changes could be seen. This type of nephritis is classified as mild chronic nephritis by Snell (1967).

(f) Cysticercus fasciolaris

This parasite was found in the liver of a 400 day old Campbell, one of the original founders of the colony. The liver contained six white cysts ranging in size from 3 to 7 m.m.s. Histological examination revealed the viable parasite with a scolex (Fig.33, V.2) surrounded by a cuff of eosinophils, macrophages and multinucleate giant cells. There was no contact between the rat colony and the adult host of this parasite, the cat, and no further cases were seen.

One 14 day old P.V.G. male control showed an eosinophil focus in the submucosa of the gut which may have been due to a parasite.

(g) Anophthalmia

A P.V.G. ♂ rat was born with an eye abnormality - the left eye was not formed. No sclera, lens, retina or cornea were present, just a central space surrounded by pigmented epithelium posteriorly and the fused eyelid anteriorly. Some golden brown pigment was present in the central cavity. The right eye was normal. This animal was perfused at 20 days (as it was thought at that stage the difference between the two halves of the cortex could be measured) which was unfortunate - with hindsight 30 or 40 days would have been a better age. The visual cortex appeared normal histologically and no difference qualitatively or quantitatively was found between the two sides. The Hardian gland and intra-orbital glands appeared normal.

(h) Peromelia

A right limb peromelia (absence of distal limb) was recorded; a P.V.G. male was born with the right hind limb missing below the femur. The other limbs were normal.

(i) Conclusion

Both strains of rats showed comparatively few lesions during the course of this work. Chronic respiratory disease was ubiquitous but was unlikely to be a complication in the quantitative C.N.S. results. The possibility of the drugs "lighting up" (Lindsey et al, 1971) latent C.R.D. infections would seem to be realised by the <30 days results (control 5/33 +ve C.R.D.: test 9/35 +ve C.R.D.) but a statistical test of significance revealed that at the 5% level there was no difference.

Overall the strains of rats showed a few differences. The Campbell rat did not breed as well as the P.V.G. and as a rule the

P.V.G. rat was heavier. The P.V.G. rat appeared no more active than the Campbell and neither strain presented any real problems in husbandry over the course of this thesis.

CHAPTER VIII

GENERAL DISCUSSION

		Page
SECTION 1.	(a) Aims of Work	184
	(b) Uses of this Investigation	184
	(c) Introduction	184
SECTION 2.	THE METHOD DEVELOPED	
	(a) Sources of Error	
	(i) Fixative	186
	(ii) Reduction Factor	186
	(iii) Section Thickness	187
	(iv) R.F. Area Error	188
	(v) Discrepancy in Counting	188
	(vi) Units Counted and Sample Size.	189
SECTION 3.	RESULTS	191
SECTION 4.	RATIONALE OF RESULTS	
	(a) Visual Deprivation	194
	(b) Cortical Effects of Retinal Dystrophy	200
	(c) The Effect of Pigment	201
	(d) Ageing Changes	203
	(e) Glial Response	204
SECTION 5.	THE EFFECT OF THE RETINOTOXIC DRUGS	206
SECTION 6.	CONCLUSIONS	207

CHAPTER VIII

SECTION 1. AIMS AND POSSIBLE USES OF THIS WORK

(a) The function of this work was to establish a method, which would allow relatively small pathological changes to be recognised. A procedure was devised utilising neuroanatomical techniques, and applied to a pathological situation which could not be distinguished from the normal by qualitative histology.

(b) This work may be of importance in toxicology, where side effects of drugs can have dreadful functional consequences and yet only produce occult pathological changes, which could be missed using qualitative methods. The advent of automatic counting equipment (Cole and Bond, 1972) can reduce the human error and the drudgery of quantitation, and make counting methods a feasible proposition.

(c) The situation chosen for this work was to compare the visual cortices of two strains of rats, the first a normal sighted strain (P.V.G.) and the second suffering from retinal dystrophy (Campbell). After the first three weeks of life the Campbell visual cortex receives negligible nervous stimulation (Dewar and Reading, 1970).

The rat was chosen for this work because although it is the most commonly used laboratory animal (Farris and Griffith, 1967), it has seldom been used as a model for visual deprivation work, and the effect of retinal dystrophy on the visual cortex of the rat has never been investigated quantitatively. In many species, visual deprivation has been produced by a wide range of techniques, from dark rearing to enucleation, but the visual deprivation inherent in animals with retinal dystrophy has rarely been exploited. The literature survey also reveals that much of the published results tend to neglect the cortex and those authors who have examined the cortex used different approaches - the number of spines (Valverde, 1968), synaptic changes (Cragg, 1967), phospholipid turnover (Wase, 1960), cell density (Mitra, 1957; Fifkova, 1967, 1969), volume of internuclear material (Gyllensten et al, 1965) and cholinesterase activity (Wase, 1960). The many variables - species used, method of deprivation, area of visual system examined and type of evaluation utilised make the comparison of findings difficult. Thus the visual cortex of the Campbell rat was an unknown quantity, although extrapolation from visual deprivation results would suggest that some change could be expected in the cortex. Biochemical investigations implied a less active cortex than the normal, as shown by a lowering of the RNA:DNA ratio and a reduced rate of RNA synthesis (Dewar and Reading, 1970, 1972).

Quantitation was chosen for this investigation and this demands standardisation of technique, for production of comparable results from individual animals. This was achieved by means of a processing reduction factor and the methods given in Chapter III. The need for standard methods is illustrated by the variation in results obtained by using different stains, the cresyl violet stained sections count was 25% higher than alternate sections which had been stained a combination of luxol fast blue and cresyl violet (Konigsmark et al, 1969).

SECTION 2. THE METHOD DEVELOPED

(a) Sources of Error

In developing a method for quantitation, the importance of recognising sources of error is important, as is attempting to reduce such errors to a minimum.

(i) Fixative - The wide variety of fixatives used by many workers in this field would suggest that none is ideal, and the graph of the effect of different fixatives (Diag.21, V.2) raises the question of the comparability of results in the literature obtained using different fixatives.

The perfused fixative used in this work is believed to cause little or no shrinkage (Schultz and Case, 1970) and none could be detected by looking for spaces between the brain and the bones of the skull. The perfused brain was used as the standard, because of the problems of immersion fixation, trauma, and the difficulty of cutting and blocking associated with fresh unfixed tissue.

(ii) Reduction Factor - The R.F. was an attempt to measure the variable shrinkage of processing. The errors inherent in this technique have been described in previous chapters. One further possible source of error is the negatives which were used to record the dimensions of the brain blocks, but repeated comparisons of the standard scale failed to show any variation. A standard photographic developing technique was used to prepare these negatives.

(iii) Section Thickness - This presented two problems - namely how repeatable is the standard thickness, and how much of the original block is present in the section? The discrepancy between the microtome setting and the measured thickness of section has been demonstrated (Graf, 1948; Marengo, 1944) and this was found in this work where a setting of 5 on the microtome gave an average thickness of 6μm (measured by direct focusing and direct measurement of cross section) (Lange and Engstrom, 1954). The most important source of error at this stage is the variation of the 6μm section thickness. Repeated measurements revealed a variation of 5% (from 5.9 to 6.2μm) despite the fact that blocks and the laboratory were kept at as uniform a temperature as possible. This source of error is highly significant in density calculations but it is unavoidable - no improvements could be made to the microtome to improve accuracy. The only solution found was to use sections which on direct cross section measurement were 6μm in thickness and to discard the small number which were outside the range 5.9 - 6.1μm. However, the sections used for quantitation, although cut at the same time as those used for direct cross section measuring could not be checked except by the inaccurate direct focusing method and the possibility that some of these sections may be of greater thickness than 5.9 - 6.1μm must be considered.

This source of error is exacerbated by the use of the $\sqrt{RF}$ to calculate the amount of the original block in the section (Diag. F, p.90, V.1). Overall the large number of sections used should reduce this error, as well as the fact that the majority of the sections quantitated for this work had a cross section thickness of just on the 6µm division.

(iv) R.F. Area Error - After cutting and staining the section was compared with the original block negative in order to calculate the R.F. The areas were magnified x50 before being drawn on graph paper for measurement of area. These measurements of area were repeated to ensure accuracy. This determination was performed just before counting to compensate for any possible contraction of the mountant D.P.X.

A further check used after the R.F. had been obtained was to use the linear R.F. i.e. $\sqrt{RF}$. The maximum horizontal (dorso-ventral) dimension of the block face was compared before and after processing to find the $\sqrt{RF}$ and this result when squared matched the area RF.

(v) Discrepancy in Counting - apart from the split cell error for which allowance has been made, can also arise from human error. To check on this, the last section counted each day was left on the microscope under oil, and the following morning this passage count was repeated. On totalling these counts and comparing with the previous result there was found to be a counting error of 1-2%, and a measuring error of 1-3%. This method of checking may be impaired by mountant shrinkage overnight, but the comparison of results showing a range of 1-3% show that this shrinkage if any must be minimal.

As a further check on sample size, and to test the counting method, litter mates of the same age and sex were processed and counted - these "doubles" are shown in the tables B, C, V.2. by the line between repeat counts. On mean cell size, the maximum variation between these comparable individuals was found to be 12%. - a lower result than the variation within an age range (e.g. 11-20 days) of 19%. Thus it would seem the method was capable of revealing family traits, within the colony.

(vi) Units Counted and Sample Size - Most of the neuro-anatomical work in this field has been concerned with the counting of neurones (Konigsmark, 1970), usually only counting those cells with a visible nucleolus, with the end result of comparing this number in different species, and relating these results to function. However, from a pathologist's point of view, the other cells, the glia are just as important, and it is deviations from the normal in all the cells in the pathological case which should be recognised. With this aim in view all the cells in the visual cortex were counted, and a sample of these cells was examined, by noting the size of long axis of the perikaryon and the longest axis at right angles to the former.

There is no hard and fast rule for sample size in this work, although sample size will depend on the degree of accuracy required and the uniformity of the structure studied. A pragmatic approach was used in this work, five passages through the cortex being counted on each individual and, as can be seen from the results, a high degree of correlation between strains was found in the 190 visual cortices quantitated. This high degree of correlation was taken to show the sample size was adequate.

The high degree of accuracy produced by the high magni-:fication and relative thinness of section required careful quantitation, and the manual counting used was a slow, painstaking tedious process. The use of automatic counting devices such as the Quantimet would have reduced the burden of counting a great deal, but such equipment was beyond the budget of this thesis.

SECTION 3. **RESULTS**

The method used produced much data which suggest in essence that up to 30 days of age there is no difference between the cortices of normal and dystrophic rats. However, after 30 days there is a clear difference in mean cell size (Fig.11, V.2). This change is diffuse and not localised as shown by dividing the cortex.

The alteration in mean cell size was shown to be due to two factors, the first an increase in absolute cell number of cells $< 79\ \mu m^2$ and the second a lowered incidence of large cells $> 147\ \mu m^2$, although there is little or no difference in the number of cells $> 79\ \mu m^2$.

The results found for total cell density, 90,000 cells/mm^3 in the P.V.G. and 118,000 cells/mm^3 compare well with those of Brizee et al (1964) 106,400 cells/mm^3, although it must be remembered that the latter applies to Area 2 of the cortex of an albino rat.

These cells were identified by means of electron microscopy and special stains on frozen and paraffin sections; the small cells to be microglia and possibly pericytes; and neurones in the case of the larger cells. Thus from day 30 the number of microglia increases in the blind visual cortex and fluctuates about a level much higher than the normal P.V.G. The number of large neurones in the blind cortex never attains the level of the P.V.G., and the decay rate from the graph (Fig. 20b, V.2) would appear to be similar.

It is postulated that for some reason microglia are stimulated to proliferate in the Campbell visual cortex, and that no other glial cells are involved. The large neurones of the visual cortex in the Campbell fail to develop to their maximum extent and this change could be due to a large number of factors. The factors which could be responsible for both these changes are discussed in the following section.

Age in Days	P.V.G. Eye.	Histology Campbell Eye.	Campbell Eye Quantitation	Mean Cell Size	Cell Density	VISUAL CORTEX Density Cells $<79\mu m^2$	Density Cells $>79\mu m^2$	Density Cells $>147\mu m^2$	Cortex Thickness
1 - 10		Normal Develop-ment	Normal Develop-ment						
11 - 20	Eyes Open				NORMAL		DEVELOPMENT		
21 - 30		Degen. Rods & Cones							
40			Retinal						
50	Develop-ing	+ outer nuclear layer	Shrinkage						
60									
70									
80	Adult Dimen-sions								
90									
100				C<P	C>P	C>P	C=P	C<P	C=P
110			Degen. + loss retinal layers						
120		Ophthalmo-scopic Differences							
130									
140									
150									
160									
170									
180									
190									
200									

C = Campbell: P = P.V.G.

SECTION 4. RATIONALE OF RESULTS

The two main findings of

(α) lack of full development of large neurones

(β) microglial response

in the Campbell visual cortex require discussion. The possible causes of these results are

(a) Visual deprivation

(b) Cortical effects of retinal dystrophy

(c) Differences between albino and pigmented strains

(d) Ageing changes.

(a) Visual Deprivation

The contention that development and subsequent maturation of neural structures are dependant upon functional activation has been investigated for more than 50 years (Riesen, 1960). The visual system has often been used to study the effects of sensory deprivation, because of the ease of deafferentation, but care must be taken in interpreting the results for comparison with the findings of this work because of variation due to

(a) technique of deprivation

(b) time of deprivation

(c) species difference

(d) part of visual system examined

(e) type of evaluation and units counted.

The "technique of deprivation" in this work is the retinal dystrophy which necessitates a control animal as the two halves of the cortex cannot be compared. The retinal dystrophy commences before the eyes are mature (Lucas et al, 1955) and it is assumed that after 21 days the cortex receives negligible nervous stimulation.

Species variation (Fifkova, 1967) limits the comparison of findings in this work with those in the literature to those only concerning rats and mice. Further reduction occurs by restricting the literature to cortical effects of deprivation.

Visual deprivation causes a variety of effects on the visual cortex on which the literature is often contradictory. Negative findings were recorded in mice after lid closing at 4-6 days, and only degeneration of the optic nerve and tract after ennucleation at the same age (Terry et al, 1962). These authors report negative results in the visual cortex in both cases using qualitative observation, and this shows how necessary quantitation is to recognise minimal changes.

However, monocular lid suturing at 14 days in Lewis rats caused a 16% decrease in the volume of the contralateral visual cortex and a 5% decrease in the deprived dorsal lateral geniculate body (Fifkova and Hassler, 1969). These authors also reported a 11.5% increase in cell number in L_4, but no consistent difference in L_5. The borders of Area 17 are indistinct, as can be seen from the variation in maps of the cortex by different authors, and volume determinations of Area 17 must be very difficult to perform. Nevertheless, in similar rats, a decrease in cortex depth was found

(Fifkova, 1967), principally due to a decrease in thickness of L_{2-4}, and this must remain a discrepancy from the results found in this work where no difference was found in cortex depth between dystrophic and control rats. The methods of deprivation and strain variation may help to account for the disagreement.

The 11.5% increase in cells, probably glia, reported by Fifkova and Hassler (1969) is in accord with the results found here, except that the dystrophic rats showed an increase in glia throughout the cortex, whereas only L_4 was affected in lid sutured rats. Again, this may be due to strain and method difference.

Dark rearing of mice (Gyllensten et al, 1965) for 20-30 days after birth produced a decrease in visual cortex thickness, and in the diameter of the nuclei of the cortex, particularly in the external layers of the cortex, L_{2-3} and L_4. No change was found in the infragranular layers, i.e. L_5 and L_6. The internuclear material per nucleus (i.e. cytoplasm and neurophil) was found to be consistently lower in all layers of the cortex.

Gyllensten et al (1965) did not use a processing R.F. and the method of quantitation was different from that used in this work. However, these findings agree in that a change in cytoplasm volume is found in all layers, but disagree on visual cortex thickness. No values for cell densities were given, nor are the cells present distinguished.

Ennucleation has also been used as a method of visual deprivation.

Tsang (1937) found that ennucleation either at birth or at 13 days in rats reduced the size of the optic nerve, decreased the volume of the lateral geniculate body and (sic) thinned the striate area, particularly L_{2-3} and L_6. This reduction in L_{2-3} and L_6 is at variance with the findings of Fifkova (L_{2-4} only) and may be explained by ennucleation and method of evaluation. Tsang (1937) further reports a difference in neurone and glia densities but gives no details.

Cragg (1967) examined the visual cortex of light deprived mice with the electron microscope and found differences in synaptic density and size. In the upper half of the cortex of blind mice the average synapse was smaller than controls, but larger in the lower half. The density of synapses was similar in the upper half, but the lower half of the cortex of dark reared mice had a lower density ofsynapses. This would suggest that exposure to light promotes the formation of new synapses of small diameter in the lower half of the cortex, and those already present in the upper half grow larger (Cragg, 1967). It would seem that stimulation is required for maturation throughout the entire visual cortex, and that changes due to deprivation should be expected not only in the upper half. No statistical analysis of synapses was carried out for this presentation.

The sites of synaptic contact have been measured in visually deprived animals, i.e. the number of spines per dendritic segment in Golgi stained sections of cortex. Valverde (1967) reported that dark rearing mice reduces the number of spines along the vertical shafts of L_5 pyramidal cells. This reduction was postulated as being due to the lack of visual input "modifying the functional and anatomical arrangement of the visual cortex". Ennucleation of mice eyes (Valverde, 1968) at birth limits this reduction of spines per dendritic segment only to the level of L_2 and L_3 and not all over the apical dendrites as occurs with dark rearing, i.e. "the varied structural changes caused by the reduction in visual input take place in all cortical layers with dark rearing" but with ennucleation only at L_4 and L_3 (Valverde, 1968). The mechanism affecting dendritic spines would appear to be different in both cases, and must be due to the different methods of attaining visual deprivation, but cannot be completely explained. These effects are called transneuronal changes - i.e. the production of slight transneuronal structural changes and should be differentiated from transneuronal degeneration, i.e. - complete degeneration of the post synaptic element. Transneuronal degeneration is dependant on the degree of deafferentation and the stage of growth for rate and extent (Riesen, 1962). In this situation, transneuronal change would also appear to vary with the amount of deafferentation, i.e. lid suturing as against ennucleation, although it seems the more minor lid suturing effects the entire cortex unlike ennucleation which only effects L_4 spines.

However, despite this inexplicable result, it would seem that the spines in the cortex are affected by visual deprivation, and it has been shown (Colonnier, 1964, 1966) that post synaptic membranes and spines attached to degenerating terminals are phagocytosed, which could be a factor in stimulating the microglial response in the Campbell visual cortex.

Deafferentation produces a much quicker structural arrest and degeneration during the foetal period (Hess, 1958). The removal of one eye from foetal guinea pigs at 46 days of gestation caused a decrease in the number of fibres in the contralateral visual cortex within four days, and also a loss of cells in the contralateral superior colliculus.

Thus visual deprivation would seem to cause changes in the

(1) Volume of the visual cortex

(2) Thickness of visual cortex

(3) Number of glial cells

(4) Nuclear diameter of all cells

(5) Cytoplasm volume of all cells

(6) Synaptic size and density

(7) Spines per dendritic segment.

The results of this work disagree in that no change in cortex thickness was found, but do agree on the change in cell density due to glia and on the change in cell size.

The opposite of visual deprivation, enriched environment, has also been shown to cause changes in the visual cortex of the rat (Krech et al, 1963; Diamond et al, 1964, 1966). The effects were an increase in cortex depth with L_2 and L_3 showing the greatest increase (Diamond et al, 1964) and an increase in glial cells, principally oligodendroglia and astrocytes. No changes were found in perikaryon size (Diamond et al, 1966). This increase in astrocytes and oligodendroglia is thought to be due to one or more of the following reasons - to supply additional neuronal nutritional needs, to support newly formed fibres, to regulate local movement in the brain, to give additional specificity to synaptic membranes, to respond to axoplasmic movement, to regulate vascular flow, to encode experience or to supply energy or substrate to the axons. In the dystrophic cortex it would seem that neither astrocytes nor oligodendroglia are involved in the changes and this accords with the results obtained above.

(b) The Cortical Effects of Retinal Dystrophy

The visual cortex of the mouse with retinal dystrophy showed at 30 days, compared with normal controls,

(i) A decrease in nuclear diameter and internuclear material (neurophil and cytoplasm) in L_{2-3} and L_4.

(ii) No change in L_5 and L_6 (Gyllensten and Lindberg,1964).

These authors used a different quantitative method and did not allow for processing variations, but nevertheless it is surprising that no change was found in L_5 and L_6. As the supragranular layers are the last to attain full differentiation, it is expected that these would be principally affected. However, electron microscopic investigations of synapses in the lower half of the cortex (Cragg, 1967) of normal and dark reared mice at 3 weeks of age suggest that the lower half of the cortex is not totally mature and the changes recorded here throughout the cortex may be due to the difference in species, in quantitation method and units counted.

Neurone density and visual cortex depth measurements on dystrophic mice (Mitra, 1957) found no difference from controls. It would appear that the method used in this work (which ignored glia) was not sensitive enough to measure the small difference in the large neurones.

These two papers are all that has been published on the histology of the cortex of the mouse with retinal dystrophy, and nothing has been published on the cortex of similar rats.

(c) The Effect of Pigment

Another factor which requires consideration is that the differences noted in the course of this work may be complicated by the fact that the P.V.G. has pigmented eyes whereas the Campbell has pink eyes. There are said to be differences between pigmented and albino animals - the eyes of albinos are larger (Donaldson, 1924) the optic nerves are smaller and have more unmyelinated fibres (Bruesch and Arey, 1942), and the visual cortex is thinner (Sugita, 1918). Sheridan (1965) found in his work on interocular transfer that albino rats showed less successful transfer after monocular training. He interpreted this as being due to a possible reduction in the uncrossed pathway in the albino. This was confirmed by comparing ipsilateral degeneration after eye removal in P.V.G. and Wistar rats (Lund,1965). These results do not agree with other workers, who suggest that the strain and not pigment may cause the variation. The eyes of both strains of rats used in this work show no difference in size and an electron microscope investigation of opticnerve in the albino showed a high or number of fibres, 117,000, all of which are myelinated

(Forrester and Peters, 1967) than the 80,100 of the pigmented rat (Bruesch and Arey, 1942), and the 74,800 of the albino found in paraffin sections. Bruesch and Arey also found 21% of the albino optic nerve fibres myelinated which again is in contradiction to the findings of Forrester and Peters.

Similarly, an anatomical comparison of visual pathways in albino and hooded rats revealed no difference (Hayhow et al, 1962), and the results in this work disagree with the findings of Sugita (1918) on the depth of the visual cortex. However, recent work in guinea pigs (Giolli and Creel, 1973) and rats (Cunningham and Lund, 1971) have again shown a reduction in the number of uncrossed fibres coupled with an abnormal distribution of these fibres and this possibility must be considered.

No quantitative work has been performed on the comparison of the visual cortices of pigmented and albino rats, and the effect if any, of the lack of uncrossed fibres on the visual cortex is conjecture. However, the possibility of a lack of pigment contributing to the differences observed in the cortices of the P.V.G. and Campbell rat should be realised. Some support for this possibility is found by comparing the results of this work with those of Brizee (1964) for total cell density in Area 2 of a white rat.

P.V.G.	Area 17	90,000 cells / mm^3
Campbell	Area 17	118,000 cells / mm^3
White Rat	Area 2	106,400 cells / mm^3

Although different areas are being quantitated, both the Campbell and the white rat appear to have a higher cell density than the pigmented P.V.G.

After this work was completed, a new strain of rats (Hunters) has been developed from crossing the P.V.G. and Campbell strains. This new strain has the pigmented eyes of the P.V.G. but suffers from retinal dystrophy like the Campbell (Reading - personal communication). The quantitation of the visual cortex of this strain would clearly be of assistance in resolving this problem.

(d) Ageing Changes

Ageing changes have been studied using the neurones of the C.N.S. as they are not replaced and do not divide (Brun and Brunk, 1973). An accumulation of heavy metals, particularly iron, was found to accompany ageing, and these metals appear at least in part, to be located in lysosomes (Brun and Brunk, 1973). In the electron micrographs of the Campbell visual cortex lysosomes appear more prominent than in the P.V.G. There is, with ageing, a further decrease in the number of neurones per volume of cortex (Brody, 1955) and cell shapes change - the cell becomes smaller and the nucleus becomes crenated or angular in outline (Schade and Ford, 1971). Lipochrome pigment is found in nerve cells and macrophages of aged rats (Steenis and Kroes, 1971) and again these were found in the Campbell visual cortex. No vascular changes of ageing (Kuhlenbeck, 1954) were seen, but the increase in glia cells in the Campbell visual cortex is also an ageing change (Brownson, 1956). Fatty vacuoles accumulate with advancing age in human nerve cells (Andrew and Winston-Salem, 1956) and although only rarely seen in the blind neurones are visible in the microglia of the Campbell.

Thus many of the findings in the Campbell visual cortex, the prominent lysosomes, the lipochrome pigment in the nerve cells and the increase in glial cells could be regarded as ageing changes. However, there were no vascular changes, and the rate of decrease of neurones shown in the graph of >200 - 500μm (Fig.20b, V.2) is similar for the P.V.G. although this method of quantitation may be too coarse to appreciate an accelerated ageing change.

(e) Glial Response

The glial reaction of microglia alone in the visual cortex of the blind rat is somewhat surprising in that astrocytes are not involved. This may be due to the relative paucity of astrocytes in the cortex, to the fact that gliosis only occurs as a result of tissue destruction, and because "the intensity of the reaction is in direct proportion to the proximity of the reacting cells" (Greenfield, 1958). No massive destruction of tissue occurs in this condition and the microglial response could be due to changes too mild to stimulate an astrocytic response. The stimulus may be due to the ageing change signs, the possible change in spines, or to defects in myelin.

Myelinisation of optic nerve fibres was retarded in mice reared in darkness, accompanied by a delay in maturation of the ganglion cells of the retina (Gyllensten and Malmfors, 1963). Changes in perikarya of neurones have been recorded during myelinisation of rat sciatic nerve - timetable, onset myelin formation day 1-6, progression day 8-15 and termination about day 112. The amount of cytoplasm in the neurone was proportional to the amount of axoplasm, and axon growth was found to be coordinated with myelin deposition (Martinez and Friede, 1970). In fact myelin formation may be controlled by axon calibre (Friede, 1972). With the "failure" of the large neurones to develop to their maximum extent this may be the reason for the myelin figures seen in the Campbell visual cortex and not in the P.V.G. However, on using myelin stained sections no abnormalities could be seen, and this illustrates the problem of slight changes where the normal methods of staining are often too coarse to show anything but massive alterations.

The last possibility to explain the glial response is that it is a direct retinal dystrophy effect on the cortex, as the disease progresses; although the findings in the rat with retinal dystrophy resemble those produced by visual deprivation in other species without any disease process.

The increased numbers of glial cells is in accordance with the biochemical findings of a significant lowering of the RNA:DNA ratio in the Campbell rat, as glia have a smaller amount of RNA than neurones (Dewar and Reading, 1970, 1972).

SECTION 5. THE EFFECT OF THE RETINOTOXIC DRUGS

The three drugs

(α) 1: 5-Di- (p- aminophenoxy) pentane hydrochloride hydrate 968A

(β) 1 - p - Aminophenoxy - 5 - phthalimidopentane 2948A

(γ) Sodium Iodoacetate

although not very effective on the rat retina (Noell, 1951; Goodwin et al, 1957) were used in this work to test the sensitivity of the method devised. No differences were found when the control P.V.G. or Campbell rats and experimental rats were compared and it is presumed that either the drugs had no effect, or the method was too insensitive. Nothing could be found in the literature on the quantitative histology of the effects of these drugs, but Ashton (1957) found that 968A had no effect on the rat eye using qualitative assessment. It would seem that these drugs are not retinotoxic in the rat.

Although Lashley (1932) found that the rat eye "represented a fairly efficient optical system, with an acuity of 1/50 - 1/25th that of man" other workers such as Kreig (1946b) have maintained that "the albino rat has very poor eyesight". Thus it would seem that despite the fact that the rat is the most commonly used laboratory animal, it is not the ideal animal for testing retinotoxicity, and other species which have superior eyesight e.g. the rabbit, would be preferable.

SECTION 6. CONCLUSIONS

The quantitative method devised in this work proved to be capable of indicating minimal changes, which were not recognisable by qualitative means. The situation chosen to test the system was the comparison of the visual cortex of the rat with retinal dystrophy and the normal, but this method could be applied to other areas, provided the basic requirements of quantitation can be met.

The differences found in the cortices of normal and rats with retinal dystrophy, were firstly an increased number of small cells, and secondly a reduced number of large cells in the blind cortex. The small cells on investigation were found to be microglia and the large cells to be neurones. The pathogenesis of these changes is not clear, visual deprivation will produce a defect in the maturation of neurones, but other factors also play a part, and it is difficult if not impossible to suggest which factors are the most important. Microglia are involved, either as a result of lack of visual stimulus, or as a result of changes produced by the visual deprivation; the latter being represented by changes in spines or myelin defects.

The drugs used in this work did not produce any pathological changes in the eyes of the rat, nor any quantitative differences in the visual cortex. The rat eye would appear to be quite resistant to such drugs and the use of other species to test for retinotoxicity is suggested.

Overall the work shows how fallible qualitative assessment of changes can be ; in fact relatively major changes are required in a localised region before the alteration can be recognised by such methods. The use of quantitative techniques may help in the study of the borderland between the normal and the grossly pathological states, and be useful in the understanding at the cellular level of the results of the opposing influences of toxic substances on one hand and body defences and repair on the other.

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CHAPTER IX

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